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(54) Title: ELECTROPHORETICALLY MEDIATED CHEMICAL ANALYSIS (57) Abstract Disclosed are methods of electrochemical analysis of an analyte in a sample. The methods include providing a sample comprising an analyte, and introducing the sample into a capillary tube containing electrophoretic running buffer and a reactant. According to the methods of the invention, one or both of the analyte and the reactant is electrically charged. Chemical contact between analyte and reactant induced by their relative electrophoretic mobility results in the breaking or formation of a covalent bond of one of the analyte or the reactant to produce a detectable product. The methods also include the steps of imposing along the length of the capillary tube an electric current for a time sufficient to bring into chemical contact within the tube analyte and reactant so as to allow formation of product, and detecting the product.		

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Electrophoretically Mediated Chemical Analysis

The invention relates in general to techniques for analysis of chemical species, and in particular to
5 analyses involving electrokinetic separation.

Background of the Invention

Capillary electrophoresis is a well-known procedure
10 for separation of chemical components. A sample solution containing molecules to be separated is placed in a length of capillary tubing containing an electrophoretic medium. Upon application of an electric field across the capillary, different
15 components within the sample migrate at distinct rates towards the oppositely charged end of the capillary dependent upon their relative electrophoretic mobilities in the electrophoretic medium. Due to the varying electromigratory rates, the sample components
20 become increasingly separated into distinct zones or groups as they progress along the capillary. At some position along the capillary, the components of the sample are detected.

25 Electrophoresis has been applied to the separation of charged materials such as proteins, nucleic acids, and cells. These separations depend upon differences in charge density, molecular size, and partitioning or complexation with a mobile phase additive. U.S.
30 Patent 5,061,361 relates to a capillary zone electrophoresis system in which a nanoliter volume of

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- sample is introduced into the capillary tube, and an electric field is imposed on the system to effect separation of the charged components. After migration along the length of the tube, the sample components are
- 5 detected via ultra-violet absorbance. U.S. Patent 5,084,150 relates to an electrokinetic method of separation in which the surface of moving charged colloidal particles is treated so as to interact selectively with the sample molecules to be separated.
- 10 An electric field is imposed on a capillary tube containing the colloidal particles and the sample to achieve separation. U.S. Patent 5,045,172 relates to a capillary electrophoresis apparatus in which electrodes are attached at each end of a capillary tube, and a
- 15 detector is coupled to the tube. U.S. Patent 4,181,589 relates to a method for separating biological cells using an electric field. The above-described U.S. patents are hereby incorporated by reference.
- 20 One object of the invention is to perform chemical reactions in a capillary so as to allow detection of a product, while utilizing only a very small volume of sample and a small quantity of analytical reagent. Two components may be mixed, e.g., a sample and a reagent
- 25 which reacts stoichiometrically with an analyte in the sample, to produce a product, and the product electrophoretically separated and measured so as to indicate the amount of analyte originally present in the sample. Another object of the invention is to
- 30 perform a chemical analysis for an analyte in a sample without finding it necessary to direct a product based on a unique direction characteristic of the product. Another object is to electrophoretically separate, product from sample and reactant quickly at a high

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voltage, without subjecting the sample to high temperature. The invention also allows for virtually instantaneous mixing of chemical components without an active process of mixing and with a minimum of dilution and diffusion of sample and/or product. Yet another object of the invention is to regenerate a capillary electrophoretic system without the need for flushing the system. Another object is to move a sample through a capillary electro-osmotically and thus without the need for a mechanical pump. Yet another object is to perform chemical analysis on multiple components of the same sample substantially simultaneously.

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Summary of the Invention

The invention encompasses methods of analysis of an analyte in a sample, and is based on the discovery that
5 analyte determination may be performed rapidly using capillary electrophoresis on exceedingly small amounts of sample by converting analyte in the sample to a product that is detectable.

10 Methods of the invention relate to a novel concept of chemical analysis in which an analyte and a reactant are brought into contact electrophoretically and a chemical reaction is allowed to occur in which a covalent bond is formed or broken and a product is
15 formed or depleted. The product is transported with a characteristic velocity in an electric field to a detector. This process, which is the basis for the analysis methods of the invention, is termed electrophoretically mediated chemical analysis (EMCA),
20 and involves three stages: a pre-reaction stage in which the zones of analyte and reactant are merged by electrophoretic mixing, a reaction stage in which a covalent bond is formed or broken and a product is produced or depleted, and a post-reaction stage in
25 which the product is electrophoretically transported to a detector and detected. During the pre-reaction interval, it is possible to separate analytes where more than one analyte is present in the sample. In the post-reaction phase, separation of the product from
30 unreacted analyte, reactant, or any other chemical species involved in the reaction may also be effected.

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Unlike traditional electrophoresis, electrophoresis utilized according to the invention exploits inherent or induced differences in electrophoretic velocities of product and/or analyte and reactant in a given electrophoretic medium in order to mix and separate these components. The electrophoretic mixing of chemical species confers a special advantage over the traditional methods in the field of chemical analysis; i.e., electrophoretic mixing of analyte and reactant is performed without substantial dilution of the chemical species contained within the zones. Under the influence of an applied potential and a chosen electrophoretic medium, a chemical species may possess a distinct electrophoretic mobility which will allow it to electrophorese essentially independently of the bulk solution. Thus, a zone of analyte and a zone of reactant, which move in the electric field with different electrophoretic mobilities, may become interpenetrated without the volumetric addition of the bulk solutions. In addition, electrophoretic mixing does not require turbulent flow to fully merge two zones. Thus, the inventive methods of chemical analysis are simpler and more efficient than conventional chemical analysis methods. At high potentials, e.g., 300-2000 volts/cm, full electrophoretic mixing of two zones can often be achieved in milliseconds.

The electrophoretic separation capability of methods of the invention allows for separation of chemical species prior to as well as after the chemical reaction. This powerful ability confers many advantages which are absent in previous methods of chemical analysis. Prior to the chemical reaction,

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chemical species that interfere with the chemical reaction may be electrophoresed away from the analyte of interest, thereby enhancing the selectivity of the reaction. Interfering reaction side-products also may
5 be separated from the detectable product following the chemical reaction. After, the chemical reaction has commenced or been completed, electrophoretic separation will effect separation of the product from the other chemical components and thus enhance the sensitivity of
10 the reaction. Multiple simultaneous chemical analyses may also be performed utilizing this physical separation process without the need for unique detectable properties in the products of each chemical reaction.

15

One of skill in the art may manipulate a range of experimental parameters of the inventive methods which allow control of the engagement and disengagement of the analyte and reactant zones, as well as selective
20 monitoring of the detectable product. As a result, analysis methods of the invention encompass any chemical reaction for the analysis of samples containing single and multiple analytes. Reactions that may be useful in the invention include catalyzed,
25 non-catalyzed, and coupled reaction systems.

One method of the invention involves introducing a reactant and a sample comprising an analyte into a capillary containing electrophoretic running buffer.
30 As used herein, "capillary" is meant to include any channel having a diameter of less than 500 microns which dissipates heat readily. One of the analyte or the reactant, or both, is electrically charged and thus moves with a characteristic electrophoretic mobility

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towards an electrically charged pole in an electric field. An electric potential is thus applied to the capillary for a time sufficient to bring analyte and reactant into chemical contact by electrophoretic motion within the capillary. The chemical reaction which produces the product involves the breaking or formation of a covalent bond of one of the analyte or the reactant so as to allow formation or depletion of product, and the product is then detected.

10

In preferred embodiments of this method of the invention, the electrophoretic running buffer may contain reactant which is distributed throughout the buffer; alternatively, the reactant is restricted to a zone which comprises only a portion of the capillary. Where the analyte is charged and the sample contains components which interfere with the chemical reaction between analyte and reactant, it is preferable to separate the analyte from the interfering components prior to chemical contact between analyte and reactant by imposing on the capillary an electric potential for a time sufficient to separate the analyte from other sample components by electrophoretic motion. In another preferred embodiment, the electric potential is applied for a time sufficient to separate the product from one or both of the analyte and reactant prior to detection.

Practice of the invention also may involve the additional step of subjecting the chemically contacted analyte and reactant within the capillary to zero potential by removing the electric potential after chemical contact between the analyte and reactant for a time sufficient to allow ongoing enhanced reaction and

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formation of increased amounts of product or depletion of product. The product is then detected. The electric potential may be re-applied to the capillary for a time sufficient to allow separation of the product from one or both the analyte and the reactant prior to detection.

The method of the invention also permits analysis of plural analytes in a sample substantially simultaneously. This method involves introducing the sample into a capillary containing electrophoretic running buffer and one or more reactants. The sample to be analyzed contains at least first and second analytes. One member of each of the following pairs of reacting components must be charged: (a) the first analyte and its corresponding reactant, and (b) the second analyte and its corresponding reactant. Thus, the component that possesses an electrical charge will be transported in an electric field and will contact the other component under the appropriate conditions of concentration, buffer, position in the capillary, and electric potential. Upon application of an electric potential along the length of the capillary for a time sufficient to bring into chemical contact components of the chemical reactions, i.e., (i) reactant and first analyte, and (i) reactant and second analyte, detectable first and second products respectively, are produced. This chemical contact results in the breaking or formation of a covalent bond of one of the members of each chemical reaction, i.e., one of the first analyte or its reactant, and one of the second analyte or its reactant to produce detectable product.

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In this aspect of the invention, the nature of the reactants is unlimited; i.e., the first and second analytes may react with different reactants to produce different products, with the same reactant to produce the same products, or with the same reactant to produce different products. In addition, the detectable products may be identical, at least with respect to the parameter used to detect them, or they may be distinguishable. If the products are identical, then the first and second analytes in the sample nevertheless can be separately determined by virtue of their different spacing caused by the differing electrophoretic mobilities of the analytes or reactants which formed them. That is, when the sample is introduced into the capillary and the electric potential applied, each of the first and second analytes is transported within the electric field at its own velocity. When each analyte encounters the reactant, product is formed or depleted in the vicinity of the analyte; product produced from each of the first and second analytes will migrate in the capillary at the same rate as all other product formed or depleted, but starting from the position where it was formed. For example, if the first analyte travels faster than the second analyte, then product formed from the first analyte will have a shorter distance to travel to the detector than will product of the second analyte. Thus, the first analyte product will travel through the detector as a peak prior to the second analyte product peak.

The chemical reactions may be allowed to occur at zero potential, and the product thus formed or depleted may be separated and detected after the electric

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potential is re-applied. Thus, after the analytes and reactant are brought into chemical contact, but before product is detected the capillary can be subjected to zero potential by removing the electric potential for a time sufficient to allow enhanced amounts of product to form; electric potential then is re-applied along the length of the capillary for a time sufficient to allow separation of the products. Performing a plural analyte analysis at zero potential allows a significant portion of the analytes in the sample to react and to form or deplete product in a restricted area of the capillary. Thus, when the potential is re-applied, the starting points for transport of molecules of product of the first analyte will be within the zone of the first analyte, and will be different from the starting points for transport of product of the second analyte zone. A zero potential step will increase the sensitivity and accuracy of this method of the invention.

20

In yet another variation within the invention, plural analytes in a sample are analyzed by allowing each to form or deplete a different product, and detecting each product by virtue of a property that is detectable on one analyte but not another. This method includes providing a sample comprising at least first and second analytes and introducing the sample into a capillary containing an electrophoretic running buffer and at least one reactant. In this method, a single reactant may react with each analyte to form or deplete a different product, or multiple reactants may be present, and each reacts with a respective analyte to produce a separate product. One of the first or second analytes or the reactant must be electrically charge so

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that, when an electric potential is applied to the capillary, electrochemical contact between the reactant and the first analyte results in formation (or depletion) of a first detectable product, and
5 electrochemical contact between the reactant and second analyte results in formation (or depletion) of a second detectable product. In each chemical contact, a covalent bond is formed or broken on the first analyte or the reactant, and on the second analyte or reactant.
10 The first and second products are then detected.

The first and second products may possess distinct electrophoretic mobilities, and thus will reach the detector at different times. Thus, the first and
15 second analytes may possess the same electrophoretic mobility in this method of the invention because, immediately upon formation of the first and second products, these products will be transported in the electric field at their distinct velocities and thus be
20 detected as distinct peaks.

One modification which is applicable to all methods of the invention described above is to restrict the area which the sample and reactant occupy initially
25 within the capillary to two separate zones of molecules. After the electric potential is applied, the zones are brought together by virtue of the differing electrophoretic mobilities of the molecules of analyte and reactant. Thus, the analyte and
30 reactant molecules come into chemical contact as the discrete zones of analyte and reactant interpenetrate.

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Product may be formed as a result of a chemical reaction between an analyte and a reactant, or a chemical component may be depleted or consumed by the reaction. Where the component is depleted by the chemical reaction, the detector will detect a decreasing amount of the component as the result of the chemical reaction (i.e., consumption of the 'product') reaches the position of the detector.

10 The invention also features a micro-electrochemical analysis device comprising means defining a capillary an electrophoresis apparatus, means for electrically connecting the capillary with the apparatus, and a reactant which converts an analyte into a detectable product by breaking or forming a covalent bond on one of the reactant or the analyte, and means for detecting the product.

The invention also features an apparatus for electrochemical analysis comprising a capillary filled with electrophoretic buffer and a reactant which reacts with an analyte to produce a detectable product by breaking or forming a covalent bond on one of the reactant or the analyte, means for imposing an electric field along the length of the capillary, and means for detecting a product within the capillary.

In yet another aspect, the invention features a capillary having a diameter of less than 500 microns and containing an electrophoretic buffer, and an analyte and a reactant spaced apart from each other, wherein the analyte and reactant are reactive when in chemical contact to produce a product by the breaking or formation of a covalent bond.

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Advantages of the invention include the following. Methods of the invention allow for rapid analysis of one or more analytes in a sample, e.g., 10 seconds. Because the volume of the capillary used in the
5 analysis is small, e.g., 10-100 nanoliters/cm, the volume of sample which is necessary for analysis according to the invention is exceedingly small; e.g., as little as 1 nanoliter may be consumed per minute of analysis in very small capillaries, and thus the
10 invention allows for less expensive analysis of a sample. Multiple analytes in a sample may be analyzed concurrently according to the invention, and only one product may be determined, or several products if desired.

15

Another advantage of the methods of the invention is that no mechanical pump is necessary to propel the chemical components through the capillary system. The components are transported through the system electro-
20 osmotically, i.e., by virtue of their electrical charge and mobility in an electric field. Thus, components which are highly charged will be transported quickly through the capillary towards the oppositely charged pole; similarly, relatively uncharged components will
25 move slowly.

The system can be designed to be self-flushing such that between runs there is a net convective flow of buffer towards one pole of the capillary due to
30 electro-osmosis. Electro-osmosis is a property which is created by a net negative or positive charge of ions on the walls of the capillary. These ions move when the electric potential is applied to the capillary, and drag the liquid in the capillary along with them.

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There is a diminishing effect of this drag; i.e., the liquid closest to the inner wall of the capillary is dragged faster than liquid in the center of the capillary. One effect of electro-osmosis is that the capillary need not be flushed mechanically between runs because the electrophoretic system continuously pumps the buffer through the capillary at a rate of 1-70 nl/min. The electro-osmotic effect may be varied by varying the charge on the capillary wall, or by varying the viscosity of the polymers which coat the inner wall of the capillary, or by the viscosity of the solution in the capillary.

The control of the parameters of the methods described herein, e.g., sample volume, reactant volume, electric potential, capillary length, electro-osmotic flow, etc., confers on one of skill in the art exquisite control over the chemical reaction itself, and thus of the sensitivity of the system.

20

Another advantage of the methods of the invention is that the time during which the chemical components are allowed to interact may be controlled by increasing or decreasing the electric potential. For example, if a chemical reaction occurs only slowly, the potential may be decreased or turned off in order to allow the chemical components more time to interact and form or consume product. Alternatively, if a chemical reaction occurs quickly, the potential may be increased so that product is separated as it is formed or depleted. This shortens the time required for analysis significantly.

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Analysis of a sample according to the invention may be performed repeatedly and routinely after the initial profile of the chemical reaction has been produced. A test for a specific analyte in a sample may be

5 performed routinely on hundreds of samples once it is known when the product peak will pass the detector relative to other components in the capillary, i.e., reactant, unreacted analyte, or contaminants. In addition, multiple analytes may be analyzed

10 concurrently according to the invention; e.g., analyte (i) may be converted by reactant (i) to product (i), analyte (ii) may be converted by reactant (ii) to product (ii), and analyte (iii) may be converted by reactant (iii) to product (iii) simultaneously.

15 Determination of products (i), (ii) and (iii), which is indicative of the presence of analytes (i), (ii) and (iii), in the same assay is easy according to the invention. More complex reactions are also analyzable according to the invention. For example, analyte (a)

20 may be converted to product (a), analyte (b) to product (b), and analyte (c) to product (a). If analytes (a) and (c) possess different electrophoretic mobilities in an electric field, then analysis of product (a) for each of analytes (a) and (c) is easily accomplished

25 according to the invention. In addition, where products (a) and (b) possess different properties for detection, these products are also easily analyzed. Thus, multiple reactions which produce the same product and multiple reactions which produce different products

30 may be analyzed according to the invention.

The invention may encompass the analysis of many different analytes using different reactants. Examples of analytes or reactants include but are not limited to

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the following. Where the analyte is an enzyme, the reactant may be a substrate for the enzyme. Conversely, where the analyte is a substrate, the reactant may be an enzyme capable of converting the

5 substrate to a product. The analyte or reactant may also include any substance which may be converted to a product stoichiometrically where quantitation of the analyte is desired, or nonstoichiometrically where

10 simple detection of the analyte is desired. Products detectable according to the invention include any substance which possesses a property detectable by any conventional means, e.g. heat, electromagnetic energy, radiation, or fluorescence. Conventional means of

15 detection are used according to the invention and are described herein.

Other features and advantages of the invention are found within the following description and the claims.

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Description

Before describing the invention in detail, the drawings will be described briefly.

5

Drawings

Figures 1(a)-(g) are schematic illustrations of the process of the invention, wherein two zones
10 (a) approach each other, (b) initiate chemical contact and begin to produce a product, (c) become superimposed, (d) interpenetrate one another, (e) begin to disengage, (f) fully disengage, and (g) move away from each other. The product, indicated by dots, is
15 simultaneously formed and electrophoretically separated from the reacting chemical species within the zones.

Figure 2 is a simulated peak shape of an analysis where $\mu_{em,p} > \mu_{em,a}$ (left peak) and where $\mu_{em,a} > \mu_{em,p}$ (right peak). The experimental parameters for
20 this profile are $\mu_{em,p} = 5 \times 10^{-4} \text{ cm}^2/\text{Vs}$; $\mu_{em,a} = 0 \text{ cm}^2/\text{Vs}$; $\mu_{eo} = 5 \times 10^{-4} \text{ cm}^2/\text{Vs}$; $k = 0.1$; $[A]_i = 0.1 \text{ M}$; applied potential = 8000 V; $l = 25 \text{ cm}$; and $L = 40 \text{ cm}$.

25 Figures 3(A)-3(C) are schematic representations of electropherograms in an EMCA enzyme assay in which (A) ES moves faster than P, and (B) P moves faster than ES, and (C) a multiple isoenzyme assay with the relative mobilities of the products being slower than
30 those of the isoenzymes forming them.

Figure 4 shows a potential program applied according to the invention in which the voltage is dropped to zero and then reapplied (top), and the
35 resulting electropherogram (bottom).

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Figure 5 is a theoretical peak shape of the determination of substrate by enzyme according to the invention. The kinetic parameters for this simulation are: $k_3 = 1$; $[E_t] = 0.0001 \text{ M}$; $K_m = 0.001 \text{ M}$; and $[S]_i =$
5 0.01 M.

Figure 6 is a schematic illustration of an apparatus useful for carrying out the methods of the invention.

10

Figures 7(A) and 7(B) show the absorbance of (A) G-6-PDH and (B) running buffer as a function of wavelength.

15 Figures 8(a-f) show an increase in absorbance of NADPH at 340 nm (a) 2, (b) 4, (c) 6, (d) 8, (e) 10 and (f) 28 minutes at zero potential.

Figures 9(a)-9(b) are electropherograms produced
20 for the analysis of G-6-PDH at (a) high potential, and (b) low potential.

Figure 10 is an electropherogram showing the accumulated peak resulting from (a) NADPH accumulated
25 before electrophoresis was initiated, and (b) NADPH accumulated just before G-6-PDH passed the detection window.

Figure 11 is an electropherogram which shows
30 accumulation of NADPH by switching to zero potential.

Figure 12 is a plot of the relationship between the peak areas of detected NADPH product and the corresponding concentrations of the G-6-PDH analyte.

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Figure 13 shows a calibration curve obtained according to the invention for the analysis of the substrate ethanol using the enzyme alcohol dehydrogenase.

5

Figure 14 shows a comparison of an assay for ethanol according to the invention versus a conventional Sigma ethanol spectrophotometric assay.

10 The invention relates to novel methods for determining the presence and/or concentration of an analyte, e.g., substrate or enzyme, in a sample by introducing the sample into a capillary electrophoretic system to initiate a chemical reaction which converts
15 the analyte to a detectable product or which depletes a detectable species. Initiation of the chemical reaction depends upon the mixing of analyte and reactant via electrophoretic migration or electro-osmotic flow, and detection of the product depends upon
20 electrophoretic transport of the product to a detector. The detection of product which is produced or depleted by the chemical reaction is an indication of the presence of the analyte. Where determination of the analyte is quantitative, the conversion of analyte to
25 product or the depletion of product will be stoichiometric. For stoichiometric conversion of analyte to product, i.e., the amount of product detected is proportionate to the concentration of analyte in the sample, measurement of the product
30 detected can be used to calculate the amount of analyte present in the sample. Methods of the invention thus allow for measurement of the presence of an analyte in a sample. In addition, the inventive methods may be

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used to measure the quantity of analyte in a sample, e.g., to determine the concentration of substrate in a reaction volume or the quantity or activity of an enzyme in a sample.

5

Capillary electrophoretic methods of the invention involve three phases for the determination of an analyte in a sample: mixing of analyte and reactant; the chemical reaction itself; and detection of a species whose production or depletion is indicative of the presence, concentration, or quantity of an analyte of interest.

The time required to perform an assay according to the invention (t_{assay}) encompasses each of the following temporal phases: the time required for the electrophoretic mixing of the analyte and reactant (t_{mix}), the time in which the chemical reaction occurs (t_{rxn}), and the time needed to transport the detectable species to the detector (t_{det}):

$$t_{\text{assay}} = t_{\text{mix}} + t_{\text{rxn}} + t_{\text{det}} \quad (1)$$

The time window available to perform the mixing and reaction phases of the analysis in order to form the product which is (t_{form}) is generally limited to the time interval between the injection of the analyte and its passing by the detector:

30

$$t_{\text{form}} = \frac{1L}{(\mu_{\text{em}} + \mu_{\text{eo}}) V} \quad (2)$$

where L is the total length of the capillary over which the potential, V, is applied, μ_{em} is the

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electrophoretic mobility of the analyte in the given electrophoretic medium, and μ_{eo} is the electro-osmotic flow of the capillary electrophoretic system.

5 1. Mixing of Reagents

The mixing of the analyte and reactant is accomplished according to the invention by exploiting differences in electrophoretic velocity between chemical species. The inventive methods require that
10 at least one chemical species, the reactant or the analyte, be electrically charged and, thus possess a characteristic mobility in an electric field. Where both analyte and reactant are charged, differences in charge density will result in differential
15 electrophoretic mobilities in an electric field. Electrophoretic mixing may be achieved where (i) the faster moving component overtakes the slower moving component while one or both are migrating towards the same electrode, or (ii) ionic components moving in
20 opposite directions electrophorese into each other during migration through the capillary.

The electrophoretic velocity of a given species is defined as the sum of a chemical species' electrophoretic mobility, μ_{em} , and the capillary
25 electrophoretic system's electro-osmotic flow, μ_{eo} . Electrophoretic mobility is dependant upon the nature of the chosen electrophoretic medium. In free zonal capillary electrophoresis system employing a buffered
30 electrophoretic medium, the electrophoretic mobility of a charged species is governed by the charge density of the substance, and is defined by the equation:

$$\mu_{em} = \frac{Ze}{6\pi\eta a} \quad (3)$$

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where Z is the effective net charge of the species, e is the electrical charge, η is the solution viscosity, and a is the hydrodynamic radius of the species.

5

Sieving medium is employed as in capillary gel electrophoresis, electrophoretic mobility is determined by both charge density and molecular dimensions. In micellar electrophoretic separations, electrophoretic mobility is dependent upon both the chemical species' electrophoretic mobility in the bulk solution and the partitioning coefficient of the species as a charged micellar additive with its own electrophoretic mobility. In various forms of capillary electrophoretic separations involving complexatory additives in the electrophoretic medium, electrophoretic mobility is determined by the charge density of the analyte as well as the degree of complexation with a charged or uncharged additive possessing its own electrophoretic mobility. The availability of different electrophoretic media allows various physical properties of analyte or reactant to be exploited so as to vary its electrophoretic velocity. This, in turn, allows the components to electromigrate at different rates in an electric field. Therefore, spatially distinct zones of chemical species can be brought into physical contact within an electric field.

30 Typically, in an assay of the invention, a zone or plug containing analyte will be merged with a zone containing reactant. As a potential is applied, the zones will migrate towards each other, as shown in Figure 1(a), at a rate dependent upon the difference in

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electrophoretic mobilities of the two components of interest in the given electrophoretic medium. At a given point along the capillary, the zones will come into contact and begin to merge (Figure 1(b)).

- 5 Simultaneously, product will begin to form, and migrate towards the detector at its own characteristic velocity. In Fig. 1, product is indicated as dots. The time required for the reagent zones to make physical contact (t_{contact}) can be calculated based
10 upon the difference in electrophoretic mobility between the two species ($\Delta\mu_{\text{em}}$), the distance between the zones (d , the distance between the leading edge of the zone of greater electrophoretic velocity and the trailing edge of the zone of lower electrophoretic mobility),
15 and the applied electric field strength (V/L):

$$t_{\text{contact}} = \frac{dL}{\Delta\mu_{\text{em}} V} \quad (4)$$

- 20 The interpenetration of the two zones will continue as the potential is maintained, and formation and separation of product continues. Equation (4) also can be used to calculate the time required for the two reagent zones to fully interpenetrate (e.g. the smaller
25 zone is completely merged within the larger zone) if d is defined as the shorter distance of that between the two leading edges or the two trailing edges (Figure 1(c)). The full interpenetration of the smaller zone within the larger zone will continue, as
30 shown in Figure 1(d), until disengagement of the zones begins (Figure 1(e)).

-24-

The time at which full interpenetration of the smaller zone within the larger zone ends can be calculated from equation (4) if d is defined as the greater distance of that between the two leading edges or the two trailing edges. The time interval during which total merging of the zones occurs (Δt_{merge}) can be estimated as

$$\Delta t_{\text{merge}} = \frac{\Delta w L}{\Delta \mu_{\text{em}} V} \quad (5)$$

where Δw is the difference in widths of the two peaks.

The zonal engagement will cease (Figure 1(f)) as the two zones completely pass by each other (Figure 1(g)). The time at which the two zones fully disengage can be calculated from equation (4) if d is defined as the distance from the trailing edge of the zone of greater electrophoretic velocity to the leading edge of the zone of lesser electrophoretic velocity.

There are numerous advantages to electrophoretic mixing over conventional mixing of bulk solutions. As molecular species electromigrate essentially independently of the bulk solution, electrophoretic mixing merges two or more zones of electromigrating components without a substantial change in volume and, therefore, without dilution of the zones. Present theory on capillary electrophoresis systems suggests that lateral diffusion is the major factor causing dilution of reagent zones. A zone of analyte can be made to engage several hundred times its own volume of reactant by allowing a small zone of analyte to migrate through a larger zone of the reactant. The ratio

-25-

(R_{vol}) of reactant volume to analyte volume encountered by a relatively narrow analyte zone as it interpenetrates a wide reactant zone is dependent upon the difference in electrophoretic mobility between the two species ($\Delta\mu_{em}$), the width of the analyte zone (w), the time over which engagement of the two zones occurs (t_{engage}), and the electric field applied (V/L):

$$R_{vol} = \frac{\Delta\mu_{em} t_{engage} V}{LW} \quad (6)$$

Electrophoretic mixing allows for two zones to be fully interpenetrated without the need for turbulent flow and resulting bandspreading. In the chemical analysis of the invention, nanoliter streams of eluent and substrate are mixed within a few seconds, and the product is transported through a laminar flow system to the detector with minimal bandspreading. Bandspreading is minimized due to quick transport of product to detector relative to the duration of the chemical reaction; i.e., as soon as product is formed, it is transported to the detector. The fast transport time may be attributed to the small size of the capillary and the large electric potential applied to the system. Typically, the capillary is 25-100 μ in diameter and 5-100 cm in length. The range of electric potential applied to the system is usually in the range of 1-300 volts/cm, but may be as high as 2000-3000 volts/cm. Shorter assay times may be achieved in a number of ways, e.g., by decreasing the length of the capillary and coordinately decreasing the voltage applied to the system, or by increasing the length of the capillary and coordinately increasing the voltage. Thus, the

-26-

inventive methods are optimized such that, once rapid electrophoretic mixing initiates the chemical reaction and product is formed, product is quickly transported to the detector. The efficiency of analysis methods of the invention, typically greater than 10^5 theoretical plates, allows for the transport and mixing of reagent zones with minimal bandspreading. The absence of significant turbulence also simplifies theoretical and experimental considerations of the analytical methods.

10 The pre-reaction phase of the inventive methods also can be used to separate different analytes of a sample which interact with the same or different analytical reagent to produce or deplete the same or similar detectable products. Differences in electrophoretic velocities of the analytes in an electrophoretic medium may be exploited by performing a separation of the analytes prior to their engagement with the zone containing the reactant. This capability allows for the determination of plural analytes which are converted to indistinguishable detectable products if the analytes themselves possess dissimilar electrophoretic mobilities in the chosen electrophoretic medium.

25

2. Chemical Reactions Useful in the Invention

The chemical reactions which may be performed electrophoretically according to the invention to analyze an analyte involve the reaction of the analyte with a reagent to produce or deplete a detectable product. The product is detectable by virtue of its unique electrophoretic mobility or electrophoretic properties. The reactions which are performed according to the invention fall within three

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categories, all of which involve the formation of a product caused by reaction between a sample analyte and a reactant, and the breaking or formation of a covalent bond which is integral to the analyte, reactant, or
5 product.

(1) Non-catalyzed reactions. Thermodynamics favor the spontaneous occurrence of a some reactions without the aid of a catalyst. For example, the analyte being
10 determined may react stoichiometrically with a reagent to produce or deplete a product which possesses a property which is more easily detected than the analyte itself. The product is detectable by virtue of a unique electrophoretic property.

15

(2) Catalyzed reactions. The energy required for activation of a chemical reaction is decreased by a catalyst, e.g., an enzyme. Some reactions occur only in the presence of a catalyst. The inventive methods
20 may be used to analyse the enzyme, substrate, or any species essential to the reaction, e.g., a coenzyme.

(3) Coupled reactions. In some chemical reactions, the reaction of analyte and reactant to
25 produce product does not produce or deplete a product which is detectable. However, product can be converted to yet another product which is detectable. For example, glyceraldehyde kinase converts glyceraldehyde and ATP to glyceraldehyde-3-phosphate (G-3-P) and ADP.
30 G-3-P in the presence of NAD is converted by glyceraldehyde-3-phosphate dehydrogenase into 3-phosphoglyceric acid and NADH. NADH has a unique absorbance at 340 nm that allows it to be quantitated in the presence of other substances in the sample. The

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presence of NADH can be correlated to the presence of the analyte in the sample (e.g., glyceraldehyde, glyceraldehyde kinase, or ATP). This coupled set of reactions allows a substance without a unique

5 chromophore to be determined spectrophotometrically by coupling the reaction to reduction of NADH. Coupled reactions may be catalyzed or non-catalyzed. Multiple coupled reactions may be performed substantially simultaneously, where different analyte and reactant

10 zones have differing electrophoretic velocities.

The separative capability of capillary electrophoresis also can eliminate the need to couple the analytical reaction to a reaction which produces a

15 species with unique detection properties. If the analytical reaction produces or depletes a product with unique electromigratory properties, the separative capability of capillary electrophoresis can allow this substance to be detected without the need to produce or

20 consume species with unique detection properties. For example, glucose is converted to glucose-6-phosphate by hexokinase with the concurrent transformation of ADP to ATP. Because none of these chemical species possess unique UV detection properties, the clinical enzymatic

25 determination of glucose requires that glucose-6-phosphate be enzymatically converted to 6-phosphogluconate by glucose-6-phosphate dehydrogenase with the simultaneous conversion of NAD to NADH. An increased absorbance observed at 340 nm resulting from

30 the production of NADH in the coupled reaction can be correlated to the amount of glucose present in the sample. Alternatively, glucose may be analyzed via its reaction with hexokinase without coupling the

-29-

conversion of glucose to a second reaction. ADP and ATP each exhibit a similar molar absorptivity at 270 nm. However, ADP and ATP possesses different charge density and, therefore, a different electrophoretic mobilities. As a result, the consumption of ATP or the production of ADP in the chemical reaction between glucose, hexokinase, and ATP may be analyzed by monitoring the electromigration of ADP and/or ATP past the detection window. The resulting ADP peak and ATP deficit peak each serve as a quantitative measure of the glucose present in the sample.

Listed in Table I are some representative examples of chemical reactions which may be performed according to the capillary electrophoretic methods of chemical analysis described herein. In each reaction shown below, different components of the chemical system may be measured. For example, in an enzymatic system, the disappearance of substrate, the accumulation of product, or the appearance or disappearance of a by-product of the reaction (e.g. a coenzyme, such as NAD or NADH) may be detected. Furthermore, the stoichiometric relationship between the species monitored and the analyte of interest allows for quantitation of various components of the reaction system. In an enzymatic system, the species monitored can be used to quantitate the substrate, the enzyme, or a cofactor.

30

This list of chemical reactions which can be performed according to the invention is not meant to be comprehensive, but merely represents some of the chemical reactions which may be performed during the course of an analysis.

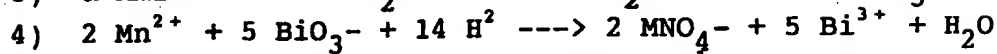
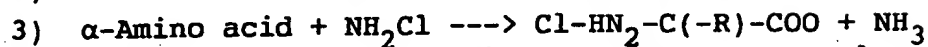
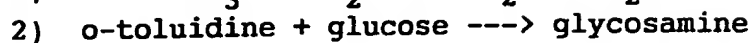
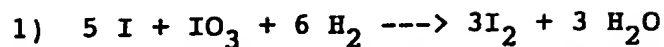
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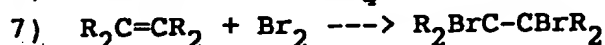
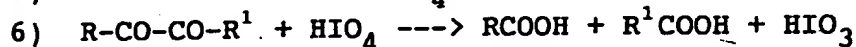
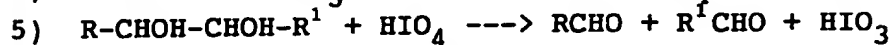
Table I

I) NON-CATALYZED REACTIONS

5



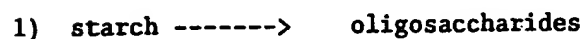
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II) CATALYZED REACTIONS

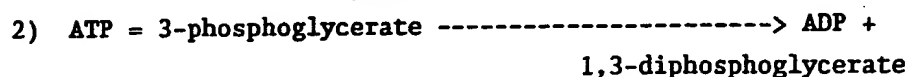
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amylase



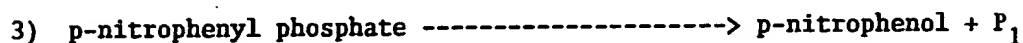
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phosphoglycerate kinase

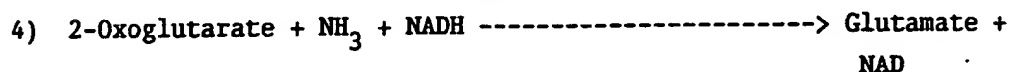


alkaline phosphatase

25



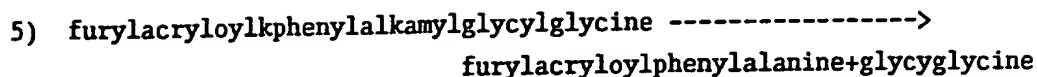
glutamate dehydrogenase



30

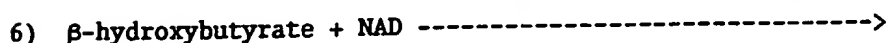
-31-

angiotensin converting
enzyme



5

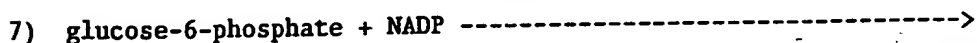
β -hydroxybutyrate dehydrogenase



acetoacetate + NADH

glucose-6-phosphphate dehydrogenase

10



6-phosphogluconic acid + NADPH

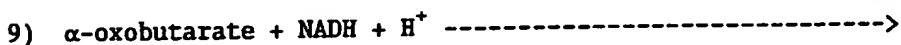
γ -glutamyl
transferase

15



L- γ -glutamylglycylglycine + 5 amino-2-nitrobenzoate

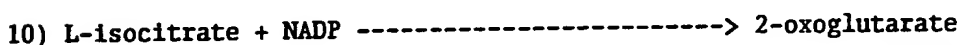
α -hydroxybutyrate dehydrogenase



20

α -hydroxybutyrate + NAD⁺

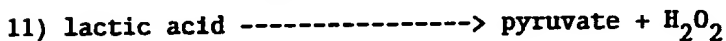
isocitrate dehydrogenase



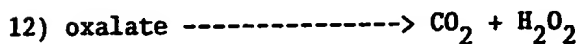
+ NADPH + CO₂

25

lactate oxidase

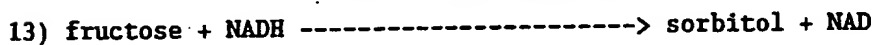


oxalate oxidase



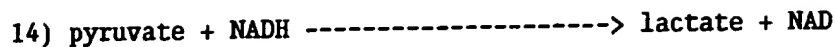
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sorbitol dehydrogenase

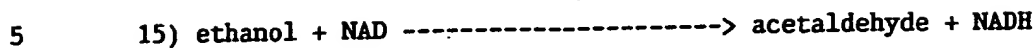


-32-

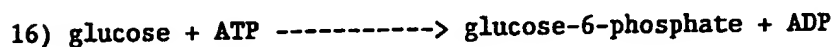
lactate dehydrogenase



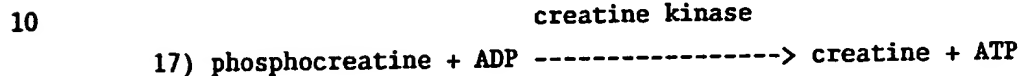
alcohol dehydrogenase



hexokinase

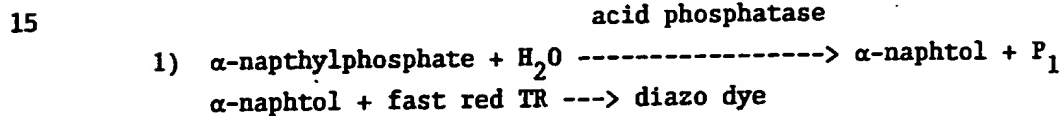


creatine kinase

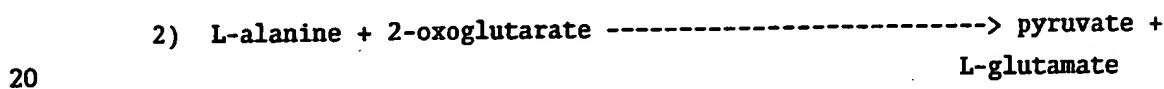


III) COUPLED REACTIONS

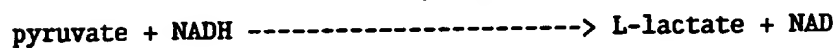
acid phosphatase



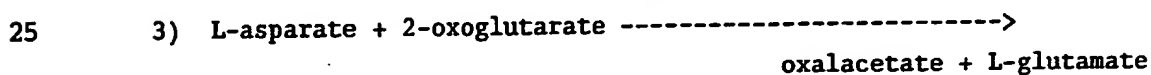
alanine aminotransferase



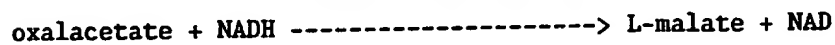
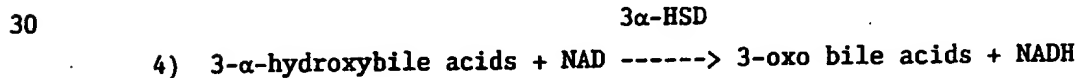
lactate dehydrogenase



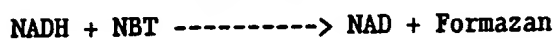
aspartate aminotransferase



malate dehydrogenase

3 α -HSD

diaphorase



-33-

cholinesterase

- 5) propionylthiocholine -----> thiocholine
 thiocholine + 5,5₁-dithiobis-2-nitrobenzoic acid --->
 5-thio-2-nitro-benzoate

5

leucine amino peptidase

- 6) L-leucyl- β -naphthylamide + H₂O ----->
 L-Leucine + β -Naphthylamine

10

hydrochloric acid

- β -naphthylamine + NaNO₂ -----> diazo reagent
 diazo + N-1-naphthylethylenediamine ---> blue azo complex

5'-nucleotidase

- 15 7) adenosine 5'-monophosphate -----> adenosine + P₁

adenosine deaminase

- adenosine -----> inosine + NH₄⁺

glutamate dehydrogenase

- 20 NH₄⁺ 2-oxoglutarate + NADH ----->
 L-glutamate + NAD + H₂O

phosphohexose

isomerase

- 8) fructose-6-phosphate -----> glucose-6-phosphate
 25 glucose-6-phosphate dehydrogenase
 glucose-6-phosphate + NADP ----->
 6-phosphogluconate + NADPH

lipase

- 30 9) triglycerides -----> glycerol + fatty acids

glycerokinase

- glycerol + ATP -----> glycerol-1-phosphate + ADP

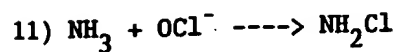
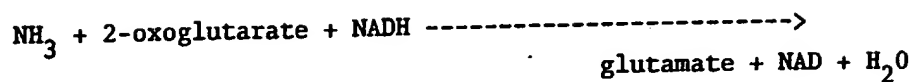
-34-

urease

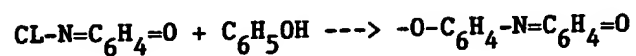
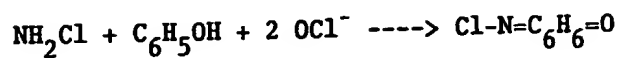


glutamate dehydrogenase

5



10



-35-

3. Kinetic Considerations

The chemical reaction performed according to the invention is governed by the kinetics of a chosen system. A simple irreversible reaction, such as

5



where A is the analyte, R is the analytical reagent, and P is the detectable product, serves as an example. An assay according to a method of the invention is typically performed with the analytical reagent in excess of the analyte ($[A] \ll [R]$) and pseudo-first order kinetics are expected as the analyte plug passes through the reagent zone. As the analyte zone engages the analytical reagent zone, A will be depleted and P will be produced at a rate directly proportional to the instantaneous concentration of the depleting A at a given point within the interpenetrated zones. The integrated pseudo-first order rate equation for an assay performed under these conditions is:

20

$$[A] = [A]_i e^{-kt} \quad (7)$$

where $[A]_i$ and $[A]$ are the initial and final concentrations of A, respectively, and k is the pseudo-first order rate constant.

25

Knowledge of the time necessary to perform the analytical reaction as well as the mixing process permits the design of an assay according to the invention. The assay can be designed in terms of separation length and applied electric field in order to minimize the analysis time, yet allow sufficient time for performance of the

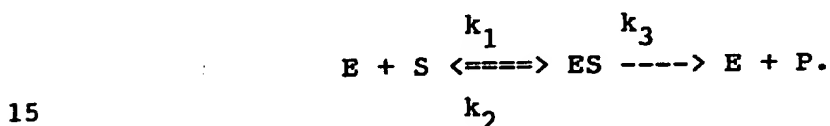
30

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assay. Using equation (7), the time of reaction (t_{rxn}) required to reach a desired quantitative extent of reaction (ϵ) can be estimated as:

$$t_{\text{rxn}} = \frac{\ln(1-\epsilon)}{k} \quad (8)$$

A second example of the reaction kinetics expected in assays performed according to the invention are those involved in enzymatic reactions. The Michaelis-Menten model accounts for the kinetic properties of many enzymes:



An enzyme (E) combines with a substrate (S) to form an enzyme-substrate complex (ES) with a rate constant of k_1 . The ES complex can either dissociate to E and S, with a rate constant of k_2 , or it can proceed to form product, P, with a rate constant of k_3 . The Michaelis-Menten equation:

$$v = \frac{V_{\text{max}}[S]}{K_m + [S]} \quad (9)$$

shows that the rate or velocity (v) of the reaction of a single substrate with an enzyme is dependent upon the maximal rate of reaction when the enzyme is saturated with substrate (V_{max}), the concentration of the substrate ($[S]$), and the Michaelis-Menten constant (K_m). V_{max} is equal to the product of k_3 (the turnover number) and the total enzyme concentration ($[E_T]$). The turnover numbers for most enzymes with their physiological substrates typically lie in the range of 1 to 10^6 per second. Defined as $(k_2 + k_3) / k_1$, K_m

-37-

values for enzymes range widely though generally lie between 10^{-1} and 10^{-7} M. The K_m value for an enzyme depends upon the particular substrate as well as environmental conditions such as temperature, pH, and ionic strength.

5

The Michaelis-Menten equation reveals that at high substrate concentrations ($[S] \gg K_m$), where the enzyme is saturated with substrate, v will approach V_{max} and remain relatively constant until the substrate is depleted or until product accumulation produces inhibition. At low substrate concentrations ($[S] \ll K_m$), the rate of the reaction is directly proportional to the concentration of the substrate. As a zone of substrate merges with a zone containing enzyme, the initial rate of the reaction will depend upon the relative concentrations of the substrate and enzyme within the overlapping region. If the concentration of substrate is high enough so that enzyme-saturating conditions prevail, a relatively constant reaction rate (V_{max}) will be observed until sufficient substrate depletion has occurred and enzyme-saturating conditions no longer exist. As the substrate is depleted, the rate of the reaction and, therefore, production or depletion of the detectable product will decrease. The rate at which this depletion of substrate occurs depends upon the relative concentrations of the enzyme and substrate, as well as the turnover number of the enzyme. This rate is also dependent upon the Michaelis-Menten constant of the enzyme for the given substrate.

30 4. Detection of Product

The detection process according to the methods of the invention is generally performed by allowing the electro-osmotic flow of the capillary electrophoretic system to transport the detectable product to the detector. As

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long as the detectable product has an electrophoretic mobility that is oriented in the same direction as the electro-osmotic flow, or is oriented in the opposite direction but is of a lower magnitude, any detectable product which is formed prior to the analyte passing by the detection window will be observable.

Because the detectable product is electrophoresed away from the reacting analyte at a constant rate (equal to the difference in electrophoretic mobility between the analyte and the detectable product), the resulting peak in the electropherogram provides a profile of the rate of the reaction. The time required for the observed detectable product to migrate to the detector (t_{mig}) can be calculated as shown in equations (10), (11), and 12. t_{mig} depends upon the time during which the analyte moves ($\mu_{\text{em},a}$) within the capillary from the point of injection (d) prior to product formation, and the time (t_{det}) required for the product to move ($\mu_{\text{em},p}$) the remaining distance ($1 - d$) to the detector window:

$$t_{\text{mig}} = t_{\text{form}} + t_{\text{det}} \quad (10)$$

$$t_{\text{mig}} = t_{\text{form}} + \frac{(1-d)L}{(\mu_{\text{em},p} + \mu_{\text{eo}})V} \quad (11)$$

$$t_{\text{mig}} = t_{\text{form}} + \frac{L - (\mu_{\text{em},a} + \mu_{\text{eo}})Vt_{\text{form}}}{(\mu_{\text{em},p} + \mu_{\text{eo}})V} \quad (12)$$

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The following equation allows for estimation of the time at which a given observed product was formed in the assay:

$$t_{\text{form}} = \frac{t_{\text{mig}} + \frac{1L}{(\mu_{\text{em},p} + \mu_{\text{eo}})V}}{1 - \frac{\mu_{\text{em},a} + \mu_{\text{eo}}}{\mu_{\text{em},p} + \mu_{\text{eo}}}} \quad (13)$$

The shape of the expected peak can be predicted based upon the kinetics of the reaction. In the simple pseudo-first order example cited previously, if it is assumed that the mixing process occurs very rapidly, such as $t_{\text{mix}} \ll t_{\text{rxn}}$, the time of formation of the product is approximately equal to the time during which the analyte is merged with the reagent zone and reaction occurs ($t_{\text{form}} \approx t_{\text{rxn}}$). This assumption allows for the following derivation of an expression for the rate of the reaction and, therefore, amount of detectable product formed with a given migration time.

$$v = k[A]_i e^x \quad (14)$$

25

$$\text{where } x = (-k) \frac{t_{\text{mig}} + \frac{1L}{(\mu_{\text{em},p} + \mu_{\text{eo}})V}}{1 - \frac{\mu_{\text{em},a} + \mu_{\text{eo}}}{\mu_{\text{em},p} + \mu_{\text{eo}}}}$$

-40-

If the electrophoretic mobility of the detectable product is greater than that of the analyte, the condition that

$$t_{\text{mig}} \geq \frac{1L}{(\mu_{\text{em,p}} + \mu_{\text{eo}})V} \quad (15)$$

exists, the first detectable product formed by a given region of analyte will be the first to reach the detector, and the kinetic effect on peak shape will be observed on the trailing edge of the peak, as shown in the left peak in Figure 2. If the electrophoretic mobility of the detectable product is less than that of the analyte, the condition that

$$t_{\text{mig}} \leq \frac{1L}{(\mu_{\text{em,a}} + \mu_{\text{eo}})V} \quad (16)$$

prevails. In addition, the first detectable product formed by a given region of analyte will be the last to reach the detector, and the kinetic effect on peak shape will be observed on the leading edge of the peak as illustrated in the right peak in Figure 2.

An experimental peak shape will be further convolved by factors such as the width of the analyte volume or plug, diffusion by the analyte and detectable species, and uneven depletion of the analyte zone due to the time required for the reagent zones to interpenetrate. For reactions with fast kinetics, the peak shape predicted by the kinetics may be obscured by these factors. However, for relatively slow reactions, the reaction kinetics are observed in the experimental peak shapes of Figure 2. Conversely, an experimentally obtained peak shape can be utilized to estimate kinetic parameters for those peaks for which

-41-

kinetic considerations dominate the peak profile. Incomplete reaction of the analyte volume may be observed if the analyte has not fully reacted with the reactant volume prior to the analyte passing by the detector, since any
5 detectable product formed after this point will not be detected. Similarly, an incomplete reaction may be observed if the volume of the reactant zone is insufficient for complete reaction of the analyte prior to the two zones disengaging. An incomplete reaction is indicated by
10 truncation of the peak obtained for the detectable product.

5. Continuous or Interrupted Electric Field

During electrophoretic mixing, contact between analyte and reactant may be transient or continuous. Transient
15 contact occurs when two narrow zones traveling at different velocities meet within the electrophoresis channel. Initially, the two zones migrate into each other and thus mix. This is followed by zone disengagement as the two species move apart due to differences in their
20 electrophoretic mobility. Product will be formed only during the time that the two species are in contact while the two zones are engaged. The chemical reaction stops after the zones disengage.

25 Continuous contact is achieved in several ways. During zonal engagement and mixing, the chemical reaction may be carried out under either zero potential (the "stopped flow" mode) or constant potential. At zero potential, when the electric field is interrupted by turning off the potential,
30 neither electrokinetic nor electrophoretic transport occurs and the reactants remain mixed in a single zone where product accumulates. This corresponds to a fixed time reaction in conventional enzyme assays. Enzymes which require longer incubation times to produce sufficient

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product for detection, e.g., a dilute enzyme preparation or an enzyme having a low turnover number, may be assayed in the zero potential mode. Although this method of continuous contact is highly sensitive, it is necessary to ascertain

5 the moment of engagement of the zones in order to interrupt the electric field. This may be accomplished by monitoring a given parameter indicative of the relative positions of the zones, or by standardizing the system such that the

10 positions of the zones are predictable given a certain length and diameter of capillary, electric potential, and analyte and reactant mobilities. If it is not possible to observe the relative positions of the sample and reactant zones, then the zones may be electrophoresed for a long enough time to allow them to pass each other, and the

15 reaction is followed by detection during the entire time interval.

Under constant potential, the analyte and reactant are mixed and separated from product continuously. Conventional

20 assays do not involve electrophoretic mixing and separation of the components of a chemical reaction. Simultaneous product accumulation and separation in conventional assays are difficult to achieve because rapid mixing of analyte and reactant may perturb separation of the product from the

25 other components. That is, mixing and separation of the sample components, the reactant, and the product will occur both during and after the chemical reaction. The methods of the invention overcome perturbation of the separation of product from reactants because both mixing and separation

30 rely on the electrophoretic mobilities of the components of the chemical reaction; i.e., rapid electrophoretic mixing of analyte and reactant initiates the chemical reaction, and the product is then electrophoretically separated from the other components. Assays carried out under constant

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potential according to the invention usually correspond to short, fixed time assays because the reaction e.g., enzyme catalysis, may occur orders of magnitude faster than the rate of separation. For example, product formation on a molar basis may be 10^2 - 10^4 time greater than the amount of enzyme, depending on the turn-over number of the enzyme and the potential applied across the zone.

Another method of continuous contact is to use a large volume of reactant relative to the volume of sample containing the analyte. In a small electrophoresis channel, e.g., in a capillary, it will take a period of time for the analyte to migrate through the relatively larger zone of reactant. The reaction of analyte with reactant continues throughout the time during which analyte migrates past the large zone of reactant. In this continuous contact method of mixing, product is formed throughout the zone of reactant and is continuously electrophoresed away from the sample zone. The product is thus more dilute than the accumulated product which formed during interruption of the electric field.

Dilution is greater with small samples and diminishes as the reactant volume becomes larger. In conventional chemical reactions, the dilution of sample which occurs during mixing of the sample and reactant generally lowers the sensitivity of detection. During chemical analysis according to the invention, sample dilution is also a function of relative volumes of sample and reactant, but only up to a certain concentration of sample, as described herein. Beyond that sample concentration, there is no dilution upon mixing.

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In electrophoretic mixing, there is an optimum sample volume which will cause little dilution and give maximum sensitivity. This sample volume will be related to the number of theoretical plates, the capillary radius, the
5 diffusion coefficient of the analyte, the viscosity of the running buffer, the electric potential, and the other variables that control bandspreading. This optimum volume may be calculated as follows.

10 Electrophoretic bandspreading theory reveals that, for a capillary column of a certain number N theoretical plates, a sample is contained in a detection volume (V_d) as it passes through the detector. This volume, V_d , is said to be independent of the sample volume (V_s), i.e., the sample
15 volume is diluted up to the volume V_d . In this respect, electrophoretic mixing is similar to other types of mixing; i.e., all methods of mixing result in some dilution of the sample. However, as sample volume V_s increases to approximately V_d , there is little increase in V_d . Finally,
20 when V_s is much larger than the volume V_d seen in analytical systems, there will be no dilution at all except on the leading and trailing edges of the peak.

The relative sizes of sample zone and reactant zones
25 also affect the speed and ease with which electrophoretic mixing may occur. Mixing of two small zones will be very rapid at high voltage. First the edges of the zones will mix, and then the two zones will be superimposed if they have exactly the same shape and volume. In general, mixing
30 will not be instantaneous. Thus, the reaction may begin before mixing is complete, depending upon both the rate with which analyte and reactant react and the dependence of the reaction rate on the concentration of reactant in the system. It is possible that product formation will not be

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constant during the course of mixing. When product is accumulated as an electrophoretic peak and transported to a detector, this peak is subject to the same bandspreading (dilution) phenomena that characterize all electrophoretic systems. At small sample volume, the bandspreading effect is greatest. As sample volume increases, there is less bandspreading.

The most convenient volumes for performing the method of the invention include combining a small volume and a large volume. For example, a small sample volume may be mixed with a large reagent volume, as follows. (Where the small volume is the reagent and the large is the sample, the procedure is essentially the same.) The capillary is first filled with the large volume, e.g., reagent; the small sample volume is then introduced at the capillary inlet. An electric field is imposed on the capillary and, after a few seconds of high electric potential (e.g., 100-300 volts/cm) the analyte and reactant will be mixed. It is then possible to vary the electric potential in order to control reaction time and allow the reaction to go to completion. For example, at zero potential, product will accumulate in a limited area and result in greater detection sensitivity. Alternatively, allowing the reaction to occur continuously, i.e., without turning off the potential will promote separation of product from analyte and reactant. Under continuous potential, product will accumulate to a lesser extent than product formed during a reaction performed at zero potential, and there will be a consequent diminished sensitivity of detection.

In contrast to mixing of two small volumes or a small and a large volume, mixing of two large volumes requires a longer time period of electrophoresis. In addition, there

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is more variability of the extent of the chemical reaction within the merging zones with two large volumes. The leading edges of two large zones make contact before the trailing edges and thus the analyte and reactant in the leading edges will react earlier than analyte and reactant in the trailing edges. This variability may be decreased by increasing the potential applied to the system. Thus, although it is desirable to rapidly mix and initiate a reaction using a small and a large volume, mixing and initiation of two large volumes is also possible if performed at a high potential.

6. Assay for Enzyme Activity or Concentration

The shape of the electropherogram obtained with the Michaelis-Menten kinetics described above depends upon the relative concentrations of the substrate and enzyme. In the assay of any enzyme according to the invention, the reactant zone (in this example, the substrate) contains enzyme-saturating concentrations of any chemical species needed for the reaction, such as substrate and coenzymes. As the analyte (enzyme) zone interpenetrates the reactant (substrate) zone, the enzyme/substrate reaction occurs, thereby producing or depleting the detectable product. If the substrate is of sufficient concentration so as to maintain enzyme-saturating conditions throughout the enzyme's electromigration through the substrate region, a relatively constant reaction velocity and thus a relatively constant production or depletion of the detectable species will be observed. As the zones first begin to interpenetrate, the quantity of detectable product will be low due to minimal zone overlap and low concentrations encountered at the edges of the zones (see Fig. 1(b)). Once the enzyme zone has fully engaged the substrate zone, a relatively constant reaction rate should be observed.

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Within the enzyme-saturating substrate zone, most of an enzyme will be sequestered in the enzyme-substrate (ES) complex, and the enzyme will traverse the substrate region with the electrophoretic mobility of the ES complex. The
5 electrophoretic mobility of the ES complex can be different than that of either E or S individually.

The highest sensitivity (i.e. greatest product accumulation) is achieved for enzymes with high turnover
10 rates and systems in which the ES complex and detectable product differ little in electrophoretic mobility. The resulting electropherogram displays a plateau representative of the relatively constant rate of the reaction as the enzyme traversed the substrate region. The maximal velocity
15 at which an individual enzyme can react with substrate is fixed by the turnover rate of the enzyme at the given experimental conditions. Variations in the height of the plateau for different injected samples of enzyme corresponding to variations in the observed rate of the
20 reaction must be related to the quantity of enzyme operating at its maximal rate. Thus, the height of the plateau is related directly to the quantity of enzyme contained in analyte injection. The plateau is observed in the resulting electropherogram until the enzyme zone passes by the
25 detector position or until the two zones disengage.

The theoretical electromigration profile of a system in which the electrophoretic velocity of the ES complex is greater than that of the detectable product is shown in
30 Figure 3(A). Because the ES complex migrates at a faster velocity toward the detector than the detectable product (P), the first product observed at the detector window (point "A" in the electropherogram) is that product formed as the enzyme migrated past the detector. In

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contrast, product detected at the other end of the electropherogram (i.e., point "B") is that product formed when the enzyme first engaged the substrate zone. The peak or spike at B is an artifact observed when a volume of enzyme is injected immediately adjacent to a region containing the substrate. This spike is the result of detectable product formed at the interface of the two zones due to diffusion which occurred between the time that enzyme is introduced into the capillary and the time potential is applied. The height of the electropherogram tracing at position "C" corresponds to the amount of product which forms while the enzyme traverses the capillary, and is proportional to enzyme concentration at constant current.

15 The electropherogram for an ES/P system in which the electrophoretic velocity of the detectable product is greater than that of the ES complex is shown in Fig. 3(B). The first detectable product to reach the detector (i.e., point "A") is that formed as the zones first merged. The last detectable species arriving at the detector (i.e., point "B") is that formed as the enzyme passes by the detection window. In either situation, if the width of injected enzyme volume is relatively narrow, the width of the observed peak represents the difference in electrophoretic mobility between the ES complex and the detectable species. Thus, the transport time of an analyte and the relative transport velocities of ES and P are easily ascertainable from the electropherogram tracings.

30 The width of the observed peak (Δt) can be related to the difference in electrophoretic mobility of the ES complex and the detectable species ($\Delta\mu_{em}$), the length of capillary

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in which engagement of the zones occurs (1), and the applied electric field strength (V / L):

$$\Delta t = \frac{1L}{\Delta \mu_{eo} V} \quad (17)$$

5

Figure 3(c) illustrates the theoretical profile obtained in the multi-analyte determination of isoenzymes in which the different ES complexes formed by two isoenzymes exhibit
10 different electrophoretic velocities. Although each reaction produces the same detectable species, specificity is obtained due to variability in electrophoretic velocity in the chosen electrophoretic medium.

15 For assays of enzyme performed in the zero potential mode, the enzyme volume is electrophoretically mixed with the substrate under applied potential, and the potential is then removed while the detectable product is allowed to accumulate for a fixed period of time. If enzyme-saturating
20 concentrations of substrate are maintained throughout the incubation period, the quantity of product produced or depleted during this time period is directly related to the quantity of enzyme operating at maximal velocity. The resulting electropherogram exhibits a peak representing the
25 accumulated product superimposed upon the plateau representing the product indicative of the applied potential periods necessary to induce electrophoretic mixing and transport of the product to the detection window. Due to the higher sensitivity achieved, the zero potential mode is
30 particularly preferred to analyze dilute enzyme solutions or enzymes having a low turnover number.

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The sensitivity of methods of the invention is inversely related to the electric field applied for a given set of experimental conditions ($\Delta\mu_{em}$, length of capillary, and separation length). This effect is of particular interest when dealing with very small enzyme concentrations in the sample of interest. The sensitivity enhancement is proportional to the turnover number of the enzyme and the amount of time the reaction is allowed to proceed in the absence of an applied potential. Figure 4 illustrates the electric potential program (top) employed in the zero potential mode and the expected electrophoretic profile (bottom).

7. Assay for Substrate Concentration

The analysis of substrate concentration according to the invention may involve an enzymatic reaction, and thus, the analyte (substrate) zone will encounter the reagent (enzyme) zone with Michaelis-Menten kinetics. At high substrate concentration, the reaction rate and, therefore, production or consumption of detectable product will be relatively constant and independent of substrate concentration. Therefore, purely enzyme-saturating conditions are of little analytical value in the determination of a substrate. However, as the substrate is depleted, so that enzyme-saturating conditions no longer exist, the rate of the reaction will decrease until, eventually, it is directly proportional to the substrate concentration. As the plug of substrate continues to pass through the enzyme zone, the rate of the reaction and, therefore, production or depletion of detectable product, will lessen, as shown in Figure 5.

Figure 5 illustrates the velocity of a reaction for a region of substrate as a function of time as predicted by Michaelis-Menten kinetics. The detectable species is

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electrophoresed away from the reacting substrate at a constant rate which is dependent upon the difference in electrophoretic mobility between the substrate and the detectable product. The resulting peak in the

5 electropherogram for the detectable product provides a measure of the velocity of the reaction at any given time within the assay. This peak will generally resemble that depicted in Figure 5. If the detectable product has a greater electrophoretic velocity than the substrate/analyte,

10 the first detectable product formed will be first to be detected, and the electropherogram will take on the appearance of a descending plateau. If the detectable product has a lesser electrophoretic velocity than the substrate/analyte, the first detectable product formed will

15 be the last to be detected, and the electropherogram will take on the appearance of an ascending plateau. The relative height and width of the peak will depend upon the relative electrophoretic mobilities of the substrate and product, the relative concentrations of the reagents, and

20 the kinetic parameters of K_m and turnover number at the given experimental conditions. The electropherogram obtained experimentally is also convolved by the width of the substrate volume, dilution of the substrate and detectable species zones, and uneven depletion of the

25 substrate volume which occurs during the interpenetration of the reagent zones.

In a substrate assay according to the invention, if electrophoretic mixing is assumed to occur relatively

30 quickly, and the enzyme zone is sufficiently wide such that the two zones will remain merged throughout the substrate's migration from the injection to detection positions, the time available for the observed reaction of the substrate is equal to the time required for the substrate to migrate from

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the injection point to the detection window as determined by equation (2). Integration of the Michaelis-Menten equation yields the time required for a given amount of substrate to be enzymatically reacted (t_{rxn}):

5

$$t_{rxn} = \frac{[S]_i - [S]_f}{V_{max}} + \frac{K_m}{V_{max}} \log \frac{[S]_i}{[S]_f} \quad (18)$$

where $[S]_i$ and $[S]_f$ are the initial and final concentrations of substrate, respectively.

10

In clinical assays of substrates, an end-point assay method is often used for determination of substrate concentration, such that the reaction is allowed to essentially reach completion prior to taking a spectrophotometric reading. In the inventive methods, substantially all of the substrate is allowed to react prior to its passing by the detection window. As a result, the descending or ascending plateau obtained eventually reaches the baseline, and the area under the curve is directly proportional to the quantity of substrate injected.

20

If the reaction is not completed prior to any remaining substrate passing by the detection window, the peak will be truncated without having returned to the baseline as the remaining unreacted substrate passes by the detection window. An attempt to extrapolate the peak to baseline may introduce error into the system due to the non-linear nature of the peak tail. This truncation effect places an upper limit on the linear range of the technique. However, the dynamic range of the method extends just up to that concentration of substrate which remains enzyme-saturating throughout its transit of the capillary. Measurement of the amount of product passing by the detector will be a dynamic

25

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measure of the quantity of substrate injected as long as sufficient substrate is depleted prior to the substrate passing by the detection window. It is desirable under these conditions to approximate non-enzyme-saturating conditions, although the resulting measurement may not be linear. While the reaction is never truly complete according to Michaelis-Menten kinetics, the time required to reach an acceptable extent of reaction, ξ , defined as the fraction of initial substrate reacted, can be estimated from equation ():

$$t_r = (1/V_{\max}(n [S]_i - K_m \log n) \quad (19)$$

Combining equations (2) and (19) allows for the calculation of the maximum concentration of substrate which can be reacted.

$$t_{\text{rxn}} = \frac{\xi[S]_i - K_m \log \xi}{V_{\max}} \quad (20)$$

Therefore, the upper limit on the linear range for a given set of experimental conditions is calculated according to equation (21):

$$[S]_i = \frac{1Lk_3[E_t]}{(\mu_{em} + \mu_{eo})V\xi} + \frac{k_m \log \xi}{\xi} \quad (21)$$

Higher concentration of substrate will result in truncation greater than that specified by ξ . Equation (21) also allows one to estimate the degree of truncation $(1-\xi)$ experienced by a given initial concentration of substrate for a set of experimental conditions. As determined by equation (21),

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the upper limit on the linear range can be expanded by increasing the separation length of the capillary or decreasing the applied electric field. Each of these methods increases the quantity of substrate which can be reacted by increasing the reaction time of the substrate in the capillary. The expansion in the linear range is accompanied by an increase in analysis time proportional to the increase in separation length or inversely proportional to the decrease in applied electric field. Another method of extending the linear range without concurrently increasing the analysis time is to increase the concentration of enzyme (and, if necessary, coenzyme) in the buffer solution. Although this does not alter the turnover number of an individual enzyme, it will proportionally increase the value of V_{\max} by raising the number of enzyme molecules capable of operating at a given turnover number.

Typically, one substrate molecule is converted to one molecule of product. Thus, detection of the presence or amount of a product as an assay of substrate in a sample is not amplifiable, as is an assay for enzyme, where an enzyme converts substrate to product multiple times. Substrates which are routinely used in clinical determinations and which may be analyzed according to the invention are, e.g., adenosine 5' - triphosphate (ATP), ammonia, bile acids, carbon dioxide, cholesterol, ethanol, glucose, lactate, oxalate, pyruvate, triglycerides, urea, nitrogen, and uric acid.

30 8. Experimental Parameters

The experimental parameters which may be varied within any method of the invention include electro-osmotic flow, electrophoretic mobility, nature of the electrophoretic medium, pH, temperature, ionic strength, viscosity, sample

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volume, electric potential, length of capillary, detection method, and the concentrations of the reacting species. These parameters may be optimized for any chemical analysis performed according to the invention. Varying one or more
5 of these parameters allows one of skill in the art to exploit a vast number of chemical analyses in the invention, and confers versatility on any method developed in accordance with the invention.

10 (a) Electro-osmotic Flow

Control of electro-osmotic flow allows for reproducible chemical analysis. Electro-osmotic flow is an inherent factor in the electrophoretic velocity of each chemical species present in the system, and affects the duration of
15 contact of the reagents as well as transport of the detectable product to the detection window. The magnitude and direction of electro-osmotic flow determines the time available for contact of analyte and reactant and for the chemical reaction, as indicated by equation (2). Consistent
20 and reproducible electro-osmotic flow is essential for quantitative analyses according to the invention, since the velocity of the species past the detection window is inversely proportional to the area of a peak. Electro-osmotic flow may be increased, decreased or reversed by
25 altering the nature of the coating of the capillary. Alteration of the viscosity of the capillary coating directly affects electro-osmotic flow by increasing or decreasing the solution drag on molecules in the system. In addition, the pH of the electrophoretic medium as well as
30 its ionic strength alter the zeta potential at the capillary/solution interface, thereby changing the solution flow.

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Electro-osmotic flow also allows for flushing of the capillary without the need for mechanical flushing. An electrophoretic system will typically electro-osmotically pump a few to several hundred nanoliters per minute of bulk solution, depending upon the diameter of the capillary and the applied potential. Thus, the capillary may be cleansed and the electrophoretic conditions for the next analysis regenerated. Electro-osmotic flushing of the system also lowers the temperature of the capillary between runs, and thus counters the effects of Joule heating at high electric fields.

(b) Electrophoretic Velocity

The electrophoretic velocity of a chemical component of the analysis is determined by its electrophoretic mobility in an electric field and the electro-osmotic flow. The electrophoretic mobility of the component is affected by the nature of the electrophoretic medium, e.g., pH, ionic strength, and viscosity. An electrophoretic medium, e.g., free solution, sieving gel, partitioning or complexatory additives, or isoelectric focusing medium, may be chosen for physical properties which will selectively impede the electrophoretic mobilities of certain components of the system. For example, a more viscous medium can increase the molecular drag of the species and, therefore, decrease electrophoretic mobility. In addition, the degree of ionization of charged molecules in the system can be selectively altered by buffering the medium at various pHs and varying the ionic strengths. Equation (4) demonstrates that a difference in electrophoretic mobility can be induced by the selection of electrophoretic medium, pH, ionic strength, and viscosity.

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(c) Viscosity

The viscosity of the electrophoretic medium may affect the diffusion coefficient for a given chemical species. Varying the viscosity of the medium is especially useful when performing assays at zero potential. As the viscosity of the medium increases, the diffusion of the components becomes less pronounced. Under zero potential conditions, increased diffusion of chemical components of the system is undesirable in that it diminishes the accumulation of detectable product. The viscosity of the medium may be modified according to any parameter known to one of skill in the art, including the following. Non-partitioning additives may be added to the medium, e.g., ethylene glycol or linear polymers. The medium may be a gel, since gels vastly increase the net viscosity of the solution, and product accumulation over an extended period of time will thus occur with little or no diffusion of the product.

(d) Volume of Sample and Reactant

The volume of sample and reactant, as well as the order in which they are introduced into the system, will be chosen in light of other experimental parameters, e.g., the relative electrophoretic velocities of analyte and reactant, the concentration of analyte or reactant within a zone, and kinetics of the chemical reaction itself. Generally, it is desirable that the zone containing a component having a higher electrophoretic velocity be introduced into the capillary later than the slower moving component zone, so that the faster component may overtake the slower component, provided the electrophoretic velocities of the components are oriented in the same direction. If the species possess electrophoretic velocities of opposite directions, the analyte and reactant zones may be introduced at different ends of the capillary such that they approach each other from opposite directions.

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The relative widths of sample and reactant plugs is chosen based on the relative concentrations of analyte and reactant, as well as the desired sensitivity of detection. For example, when injecting a small zone of highly concentrated analyte, it is desirable to use a very large volume, e.g., extending the length of the capillary, of reactant. Thus, the analyte may be quantitatively reacted with reactant as it encounters many times its own volume of reactant (see equation (6)). Typical analyte/reactant volume ratios are 1/10, 1/100, or 1/1000, etc., with use of a larger reactant volume resulting in an increase in the proportion of reacted analyte. Another example under which a larger volume of reactant is preferred is where the reaction kinetics are very slow compared to the rate of zonal traversal of the interpenetrated reagents. Conversely, if the reactant concentration is high relative to the analyte concentration, a small plug of each may be injected to perform the chemical analysis according to the invention. A small analyte or reactant volume may also be used where the time of the chemical reaction is fast relative to the time the zones are interpenetrated.

(e) Applied Potential

The potential required to impart electrophoretic motion is typically applied across the capillary by a high voltage source operated at electric field strengths generally ranging from several hundred volts per centimeter to several thousand volts per centimeter. See U.S. patents 4,865,706 and 4,865,707 hereby incorporated by reference. The application of the potential can be controlled either via manual operation, a waveform generator, or computer control.

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The rates of migration of chemical species in capillary electrophoresis are directly proportional to the electric field applied due to electrophoretic and electro-osmotic effects. Although the strength of the electric field strength does not affect the relative rates of migration of chemical species, as is evident from equations (2), (4), (5), and (6), the assay time, the time at which zonal engagement occurs, and the total time of interpenetration and, therefore, reaction are dictated by the applied potential. Once the analyte and reactant zones become engaged, the applied potential determines the nature of the contact: dynamic at potentials greater than zero and static at zero potential. As used herein, low potential refers to approximately 1 to 100 volts/cm; high potential refers to approximately 100 to 300 volts/cm. Lower electric field strengths result in slower movement of chemical species, thereby increasing the contact time of two zones. Furthermore, if the detectable species possesses a different electrophoretic mobility than the analyte (or the transient enzyme-substrate complex at enzyme-saturating conditions in an enzymatic assay), lower potential will proportionally decrease the rate of separation of detectable species from the vicinity of the reaction. As a result, low potentials offer the greatest sensitivity. One example of low potential is the zero potential mode in which maximum detectable product accumulates and, thus, higher sensitivity.

Higher potentials offer the advantage of speed as the magnitudes, but not relative values, of the rates of migration of the species involved in the assay increase proportionally. High potentials also offer the ability to mix zones rapidly and, for those reactions for which sufficient reaction time and sensitivity are not a concern,

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to minimize analysis time. Given knowledge of the demands of a given chemical system involved in an assay, one of skill in the art may choose the potential so as to optimize each of the stages involved in the assay. For example, the zonal merging stage may be done at high potential to induce rapid, uniform mixing. However, the ensuing reaction phase may be done at lower potential to allow sufficient time for the reaction to occur and to provide maximum sensitivity. The potential may then be increased to sweep the detectable species past the detector and minimize analysis time.

It is desirable to initiate a chemical reaction by mixing at a rate other than the rate at which the chemical reaction occurs, i.e., the rate at which product forms. Thus, the rate of mixing should be performed either at a substantially higher or lower rate than the rate of the reaction. The rate of mixing of sample and reactant zones may be controlled by varying the electric potential applied to the capillary. For example, when a reaction occurs rapidly, it is desirable either to mix very rapidly so that product formation is uniform across the sample zone, or very slowly so that product formation is complete across the zone as zone engagement occurs. Electrophoretic mixing occurs faster at high potential, and thus mixing at a high potential is preferred. At several thousand V/cm, mixing will occur in milliseconds. The potential then may be decreased or interrupted after mixing to allow the reaction to occur. In addition, because the time interval at high potential is short, little heat is produced.

A unique feature of electrophoretic mixing relative to mechanical mixing methods is that zones are merged by differential rates of migration. Since concentration is always discontinuous across a zone in an electrophoretic

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system, the concentration of reactants will vary during zonal engagement. A constant rate of engagement of different areas of a zone is desirable. When the zones are gaussian, leading and trailing edges of the peaks will be of lower concentration than the center of a peak. As the zones are electrophoretically engaged, the zones of low concentration are the first to mix. Thus, the reaction starts at low concentration and the rate of product formation will not be constant across a peak. To simplify interpretation of data, it is desirable to rapidly engage the zones in order to obtain a rate of product formation which approximates a constant rate. For example, when a capillary is filled with reactant and then a small sample containing analyte is introduced, the leading edges of the zones engage slowly relative to the trailing edges, which engage quickly. If the applied potential is abruptly turned off, the leading edges will be electrophoresed past each other while the trailing edges of the zone may then make contact under zero potential. Thus, the relative positions of the zones should be carefully monitored.

(f) Length of Capillary

The length of capillary used in combination with the applied potential determines the strength of the electric field and thus also affects the rates of migration of each chemical species. In addition to the overall length of the capillary, the separation length, i.e., the length between the point of introduction of the analyte into the capillary and the position at which the product passes by the detection window, is another parameter which affects the assay. The separation length affects the time available to perform the mixing and reaction phases of the assay, as shown in equation (2). For slower reactions performed under uninterrupted potential, a longer separation length is often

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necessary to allow sufficient time for the reaction to occur before the analyte passes the detector.

The incorporation of a spacer (zone containing non-
5 reactive medium placed between analyte and analytical
reagent zones) also allows for the experimental regulation
of the pre-reaction separation of analytes. This option is
of importance in systems where many analytes within a sample
possessing characteristic electrophoretic velocities react
10 with the analytical reagent(s) to form the same detectable
species or species with very similar electrophoretic
velocities. Within this scheme, the analyte molecules may
be allowed to separate into distinct zones which will
encounter the reagent zone(s) at spatially and/or temporally
15 distinct location within the capillary. As a result, the
detectable species will be formed at unique locations and/or
times within the reaction chamber and, therefore, they will
be observed at the detection window at a time indicative of
the particular analyte responsible for their formation.

20

(g) Alteration of Kinetics

Kinetic parameters may be altered by the selection of
factors such as pH, ionic strength, viscosity, and
temperature. Reaction rates are highly dependent on
25 temperature, as described by the Boltzmann Distribution and
the activation energy required for a given reaction. The
use of a thermostated capillary electrophoresis system
allows for the selection of a reaction temperature.
Furthermore, the pH and ionic strength of the
30 electrophoretic medium may be varied to determine the
direction and rate of reversible reactions. Most enzymatic
reactions are reversible and thus demand optimum pH and
ionic strength ranges for maximum turnover of analyte
substrate. For reactions which are diffusion controlled,

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the viscosity of the electrophoretic medium may determine the maximal availability of reactant to analyte and, therefore, overall rate of product formation.

5 Viscosity, pH, and ionic strength also may be used to enhance the sensitivity of methods of the invention by concentrating species at the interface of two zones of different viscosity, pH, or ionic strength. Chemical species may be "stacked" at such interfaces by virtue of
10 their differing electrophoretic mobilities in the two adjacent regions of viscosity, or due to the variability in electric field between the two regions of viscosity. The principles of isotachophoresis may be used in this manner to concentrate samples in capillary electrophoresis systems
15 (Chien and Burgi, 1991, J. Chromatogr. 559: 141; Aebersold and Morrison, 1990, J. Chromatogr. 516: 79).

(h) Electrophoretic Medium

The electrophoretic medium is critical in the EMCA as it
20 is responsible for exploiting physical characteristics of the reagent species in order to impart the variability in electrophoretic velocity necessary to perform the physical processes involved in EMCA, as previously described. Those electrophoretic media employed in EMCA parallel those
25 utilized in capillary electrophoresis. Each offers the capability of exploiting unique physical parameters of the reagent species. These electrophoretic media include, but are certainly not limited to, free solution, gels, complexatory agents, partitionary additives, and ampholytic
30 species.

Free solution electrophoresis typically is performed in a buffered medium. Electrophoretic mobility in free solution is determined by the charge density of the given

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species, as previously noted in equation (3). The parameters of pH and ionic strength are determined by the identity and concentration of the chosen buffered solution. The buffer may alter the degree of ionization of various moieties contained within the chemical components, and thus their electrophoretic mobilities. The electrophoretic medium also affects the zeta potential at the capillary surface and the resulting electro-osmotic flow. The type of electrophoretic medium chosen by one of skill in the art allows for control over the electrophoretic velocities of chemical components of the system. This control extends over the physical processes involved in the methods of the invention. A myriad of inorganic, organic, and biological buffers throughout the accessible pH range have been utilized in capillary electrophoretic systems. Free solution electrophoretic systems have been used to impart variable electrophoretic mobilities in all types of molecules capable of acquiring a charge, including inorganic, organic and biological molecules of numerous classes.

Gel capillary electrophoresis imparts variability in electrophoretic mobility which is based on a chemical species' charge and molecular dimensions. This phenomenon offers a unique selectivity in the control of the physical processes involved in the inventive methods. Gels most commonly used in capillary electrophoresis are polyacrylamide and agarose gels, and can be used to analyze molecules such as peptides, proteins and DNA fragments.

The use of complexatory agents in the electrophoretic medium offers the selective interaction of charged or uncharged reagent species with charged or uncharged solution additives. One of skill in the art may choose a given

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additive based on its ability to form a complex with an analyte. The complex will then migrate in the electric field with a characteristic mobility. For example, crown ethers and cyclodextrans have been used as additives capable
5 of selectively complexing with chiral compounds.

The use of partitioning media, e.g., the addition of ionic surfactants to produce micellar solutions, provides another method of fine-tuning the analytical methods of the
10 invention. The selective partitioning of species into charged micelles having their own characteristic electrophoretic mobilities allows for the development of differential velocities for neutral species which have no characteristic velocity in free solution. Numerous ionic
15 surfactants have been utilized, including sodium dodecylsulfate (SDS) and bile salts. Micellar phases have been utilized in the analysis of species such as amino acids, drugs and drug metabolites.

20 The addition of ampholytic molecules to the electrophoretic medium provides a method of fine-tuning an analysis in which the analyte, reactant and product possess only small differences in electrophoretic mobility. Ampholytic molecules are also useful where the species
25 involved in the reaction migrate to a point in the capillary where electronic neutrality exists and, therefore, electrophoretic mobility ceases. The resulting heterogeneous solution can then proceed past the detector.

30 9. Multi-Analyte Determinations by EMCA

Methods of the invention allow for the substantially simultaneous determination of plural analytes in a sample. Thus, for example, a blood sample may be analyzed simultaneously for many different analytes. The different

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analytes may possess similar or different electrophoretic velocities, and the plural analytes may move in the electric field with the same or different velocities as the product formed after contact with one or more reactants. Examples
5 of analyses of plural analytes in a sample are as follows.

Analytes A, B, and C react with reactant R to produce or deplete product P. P may be substantially identical for each chemical reaction involving analytes A, B, and C with
10 R. Alternatively, P may be different for each reaction, but may move at the same rate in the electric field and thus be indistinguishable in terms of its electrophoretic velocity.

15
$$\begin{aligned} A + R &\rightarrow P \\ B + R &\rightarrow P \\ C + R &\rightarrow P. \end{aligned}$$

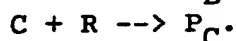
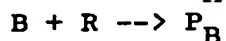
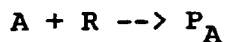
Analytes A, B, and C in this example may be distinguished if they possess different and known relative electrophoretic
20 mobilities in the chosen electrophoretic medium. Their distinctive electrophoretic mobilities may be taken advantage of by allowing for electrophoretic separation of the three analytes prior to their engagement with R. Thus, after each separated analyte, A, B, and C, undergoes a
25 chemical reaction with R to produce P, three distinct zones of P will be produced. As each zone of P migrates past the detector, the amount of P detected can be related to the concentration of each of A, B, and C present in the sample.

30 Analytes A, B, and C may react with the reactant R to produce or deplete three different products P_A , P_B , and P_C .

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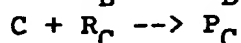
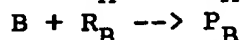
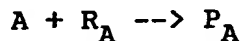
These products either possess a unique detection property or a unique electrophoretic mobility.

5



10 In this example, a pre-reaction separation of analytes is not necessary, nor is it required that the analytes possess different relative electrophoretic mobilities. The distinct electrophoretic detection properties or mobilities of each of products P_A , P_B and P_C must be known. The electrophoretic mixing of analytes A, B, and C with reactant R and the chemical reactions may occur simultaneously and/or
15 at the same rate. As each product migrates past the detector, its detected quantity will correspond to the concentration of the corresponding analyte in the sample.

20 Analytes A, B, and C may react with reactants R_A , R_B , and R_C to produce or deplete products P_A , P_B , and P_C .



25

In this example, if the three products do not possess unique detection or electrophoretic properties, then the analytes must possess differing relative mobilities in the electric field and a pre-reaction separation of analytes must be
30 performed. If the analytes migrate at the same rate in the electric field, then they may be analyzed simultaneously if the products possess distinct relative electrophoretic mobilities or distinct detection properties.

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10. Apparatus

In Fig. 6, the apparatus 10 includes a capillary 12 with an inlet end 14 and an outlet end 16. The capillary may be a pulled glass tube or any means for electrophoresing micro-
5 samples. A charged electrode 18 is in communication with conductive medium 26 and an oppositely charged electrode 20 with conductive medium 30. Electrodes 18 and 20 are electrically connected to a power supply 22. Apparatus 10 also includes a fresh supply of conductive medium 26 which
10 is fed into inlet 14 of tube 12 via conduit 24. The buffer fills the tube and exits through conduit 28 into reservoir 30. The capillary may have a diameter in the range of 1-500 μm , preferably 25-200 μm , and most preferably 75-100 μm . The length of the capillary may be in the range of 1-500
15 centimeters from the inlet end to the outlet end, preferably 5-100 cm. Capillaries which operate under electric fields of from several hundred volts per centimeter up to several thousand volts per centimeter or more are preferred. See U.S. Patents 4,865,706 and 4,865,707, hereby incorporated by
20 reference.

Output from detector 36 is connected to a recorder 44 which is connected to a computer 46. Recorder 44 records data as an electropherogram 48. Sample 34 is introduced
25 into tube 12 via conduit 32. Detector 36 detects chemical components which pass through the tube, e.g., at position 38. Detector 36 may be any detector capable of sensing components of the sample as they traverse capillary tube 12, with which the detector communicates. Detector which
30 operate by sensing change in electromagnetic radiation, such as ultraviolet, infrared, and fluorescence detectors, require that any protective coating of organic material present on capillary tube 24 must be removed over the area of the capillary on which the detector is to operate.

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Another on-column method of detection involves detection of a radioactively-labeled component of the reaction, utilizing an appropriately-designed radioactive sensing device. This detection approach could also be employed with
5 off-line collection and subsequent determination.

It is also possible for the detector to operate by directly sensing separated sample constituents as they exit capillary tube 12. Examples of such detectors include mass
10 spectrophotometric detectors and electrochemical detectors. In mass spectrometric detection the outlet end 16 of capillary tube 12 is placed in very close proximity to the inlet of a mass spectrometer and an electrode is also placed in close proximity to this capillary end, in contact with
15 the conductive medium exiting the capillary. The field across the capillary is established between this electrode at the capillary's end and the corresponding electrode or wire making contact with the conductive medium in which the capillary tube inlet end 14 is in contact. In a further
20 alternative detector embodiment, useful where a detector or probe must come into contact with the conductive medium exiting capillary tube 12, the exit end of the capillary tube is connected to a very short additional piece of capillary tube by means of a porous glass sleeve, and this
25 sleeve is immersed in conductive medium. Electrical contacts for the imposition of the electrophoresis field are made with the reservoirs of conductive medium at the respective ends of the capillary tube, and the detector probe is placed in contact with the conductive medium
30 exiting the capillary extension.

An optional programmable computer may communicate with and directs input into an optional waveform generator to control applied potential. The computers may be a single

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device or two independent devices. The generator may in turn be in communication with and direct input into power supply 22. Electrical output from power supply 22 travels along electrode 18 to conductive medium 26, or along
5 electrode 20 to conductive medium 30.

The programmable computer may be programmed to direct the waveform generator to produce the amplitude or frequency desired in a given electrophoretic chemical reaction. The
10 computer may also direct the movements of an optional automatic sampler, which in turn may direct input or output of the sample to or from the electrophoretic system. For example, the sampler may shunt the inlet end 14 between sample 34 and medium 26 so as to allow sample or medium into
15 the capillary tube.

The waveform generator may be any conventional waveform generator (e.g., Model 75, Wavetech, San Diego, CA). The waveform may be run by computer control or in stand-alone
20 mode. It is preferably capable of providing a variety of waveforms, including sine waves, square waves, triangular waves, etc., in a positive or negative sense relative to a defined reference voltage.

25 In operation, apparatus 10 may be used as follows to detect an analyte. For example, where the analyte being determined is the activity of an enzyme, sample containing the enzyme 34 is introduced into capillary 12 at inlet 14 by electrophoresis or siphoning through conduit 32. the
30 substrate is contained within capillary 12. Enzyme and substrate in this example possess charges and electrophoretic mobilities which orient them in opposite directions in the capillary when an electric potential is applied. Power supply 22 is turned on and adjusted so as to

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deliver an applied potential to the capillary via electrodes 18 and 20. The enzyme and substrate thus begin to migrate in the electric field and product is formed and begins to move toward detector 36, as shown in Fig. 1. When the product reaches detector 36, a signal is transmitted to recorder 44 and computer 46. Electropherogram 48 is then produced, mixed with the reagents in capillary 12 electrophoretically; i.e., each charged component in the tube migrates along the tube according to its charge and thus mixes with other components which migrate at a different rate.

Rapid mixing may be achieved using a post-reaction detector (El Rassi, 1976, Journal of Chromatography 559:367) as in liquid chromatography systems (Schlabach et al., 1978, Clinical Chemistry 24:1351; Snyder, 1976, Journal of Chromatography 125:287; Deelder et al., 1977, Journal of Chromatography 125:287). For example, substrate may be added to the system through a mixing-tee (e.g., where conduits 32 and 24 join in Fig. 1) at the inlet end of the capillary tube, and the reaction mixture is then pumped into the capillary tube. Product detection is achieved near the outlet end of the capillary using any conventional capillary electrophoresis detector. Because the transit time between the mixing-tee and the detector is constant, this system may approximate a fixed time assay. Enzyme activity or substrate concentration may be assayed by electrophoresing the product of an enzymatic reaction involving enzyme and substrate to the detector where it is detected. As described herein, under constant potential, the transport velocity of enzyme and product is generally different, and the product may become separated from the enzyme immediately after it is formed.

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11. Preparation of Capillary

The capillaries employed in the invention are typically constructed of silica which is coated on the outer surface with an agent, such as polyimide, to prevent breakage due to the fragile nature of silica. While there are no inherent limitations on the lengths or inner diameters of capillaries employed in the invention, typical lengths range from 5 to 100 cm. A capillary tube having a diameter of between 10 and 500 μ is useful in the invention.

10

In the methods of the invention, it is frequently advantageous to use capillary coatings. These coatings offer several advantages to the use of uncoated silica. The ionization of silanol groups produces a negatively-charged silica surface. Positively-charged analytes, such as proteins, adsorb to the negatively-charged wall thereby altering the zeta potential at the silica/solution interface. Disruption of the zeta potential alters the electro-osmotic flow and may decrease reproducibility and reduce recovery of product. Variability in electro-osmotic flow is particularly detrimental in quantitative analyses because the area of a peak observed in capillary electrophoresis is inversely proportional to the electrophoretic velocity of the species. Any variation in electro-osmotic flow will produce a concurrent alteration in peak area. Capillary surface modification may also be useful for controlling electro-osmotic flow. The ability to regulate the electro-osmotic flow serves as a powerful tool for carrying out in the invention. The process of electrophoretic mixing of zones as well as the transport of the detectable product to the detector is dependent upon the electrophoretic velocities and, therefore, the electro-osmotic flow of the system.

30

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Those coatings which have been employed in capillary electrophoresis include the covalent modification of the silica surface as well as the use of buffer additives to dynamically modify the capillary wall. Representative examples of the covalent modification technologies include epoxy polymers (Towns et al., 1992, J. Chromatogr. 599: 227), polyethylene-imine (Towns and Regnier, 1990, J. Chromatogr. 516: 69), aminopropyl-sialylated coatings (Moseley et al., 1991, Anal. Chem. 63: 109), polyacrylamide (Cobb et al., 1990, Anal. Chem. 62: 2478; Hjerten, 1985, J. Chromatogr. 471: 429). Representative examples of the use of dynamic coatings include amine additives (Lauer and McManigill, 1986, Anal. Chem. 58: 166; Nielsen et al., 1989, Anal. Biochem. 177: 20), cationic polymers (Wiktorowicz and Colburn, 1990, Electrophoresis 11: 769), and a cationic fluorosurfactant (Emmer et. al., 1991, J. Chromatogr. 547: 544). Covalent modification coupled to adsorbed dynamic coatings has also been utilized, such as in the use of nonionic surfactants adsorbed to silane-derivatized surfaces (Towns and Regnier, 1991, Anal. Chem. 63: 1126).

12. Detectors and Detection Methods

Any conventional method of detection may be used in the invention, including those used in conventional capillary electrophoresis methods. A detection method may be chosen which allows for detection of any physical property of a chemical species. These detection systems include, but are not limited to, absorbance of ultraviolet or visible radiation, fluorescence, refractive index, Raman, mass spectrometry, electrochemical, and conductivity. Detection of the electrophoretically transported product may occur at a discrete position along the length of the capillary, off-line, or by imaging the entire length of the capillary (Wu et al., 1992, Anal. Chem. 54: 219), hereby incorporated by reference.

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14. Injection Methods

5 The sample or reactant volumes may be introduced by any of the methods employed in capillary electrophoretic systems, including hydrodynamic, electrokinetic, vacuum, injection port, and syringe methods. Furthermore, the system can be readily automated for injection with commercially available autoinjectors.

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Preferred Embodiments of the Invention

The invention features an ultramicro method for performing chemical reactions in order to assay an analyte in a sample, e.g., enzyme activity or substrate concentration in a sample. The enzyme may be present in the sample, and the conversion of substrate monitored as an indication of the presence of enzyme in the sample. Alternatively, the substrate may be present in the sample, and enzymatic activity (e.g., the utilization of cofactor or the inactivation of enzyme) may be monitored to indicate the presence of substrate. An assay for enzyme activity is carried out under saturating concentrations of substrate, whereas an assay for substrate concentration is carried out under saturating conditions of enzyme. The assay may be performed in a deactivated fused silica capillary in a capillary electrophoresis apparatus, such as that shown in Fig. 6.

Examples

The results presented below demonstrate that small quantities of enzyme or substrate may be detected in a capillary zone electrophoresis system by performing the enzyme assay in the capillary according to the methods described herein. The assays rely on the differing transport velocities of the enzyme(s), reagent(s), and product(s) under applied potential, a characteristic which may be used to both electrophoretically mix the reactants and electrophoretically separate the enzyme and substrate from product. Product is transported through the capillary tube until it reaches the detector, where its concentration is determined and related to the activity or concentration of enzyme in the sample or the concentration of substrate in the sample. The detection limit according to the method of the invention appears to be three orders of magnitude more sensitive than the detection limit of conventional assays.

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1. Analysis of the Enzyme Glucose-6-phosphate dehydrogenase

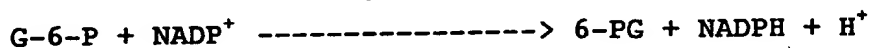
The enzyme Glucose-6-phosphate dehydrogenase (G-6-PDH, EC 1.1.1.49) may be used as a representative enzyme for analysis according to the invention. G-6-PDH has been found
 5 in almost all animal tissues and microorganisms, and catalyzes the first reaction in the hexose monophosphate shunt pathway. The clinical biochemistry of G-6-PDH deficiency has been extensively reviewed (Yoshida, 1973, Science 179:532).

10

Glucose-6-phosphate dehydrogenase (G-6-PDH), also referred to as D-glucose-6-phosphate:NADP oxidoreductase, was chosen to examine ultramicro enzymes assays because this enzyme may be readily assayed spectrophotometrically (Sigma
 15 diagnostics procedure No. 345-UV, 1990). G-6-PDH oxidizes glucose-6-phosphate (G-6-P) to 6-phosphogluconate (6-PG) while reducing nicotinamide adenine dinucleotide phosphate (NADP) to its reduced form NADPH in the presence of G-6-P.

20

G-6-PDH



The absorbance spectrum of the product NADPH is uniquely different than that of either the G-6-PDH (Figure 7(A) or
 25 the running buffer (Figure 7(B)). Thus, this spectrum may be used to monitor the reaction (Sigma diagnostics procedure No. 345-UV, 1990). NADPH has an absorption maximum at 340 nm ($\epsilon = 6.22 \times 10^6 \text{ cm}^2/\text{mole}$). The enzyme 6-phosphogluconate dehydrogenase (6-PDH) can interfere, such
 30 as in serum samples contaminated with 6-PGDH from erythrocytes which contain no G-6-PDH. In the presence of 6-PGDH, 6-phosphogluconic acid may be further oxidized to produce a second mole of NADPH. Addition of maleimide to the incubation mixture will inhibit 6-PGDH. Fig. 8 shows an
 35 increase in absorbance at 340nm of NADPH after (a)2, (b)4, (c)6, (d)8, (e)10 and (f) 28 min. at zero potential.

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The method of the invention described in this example is be carried out using any conventional capillary electrophoresis system, e.g., those described in the U.S. Patents incorporated by reference above. A preferred capillary electrophoresis apparatus is an ISCO 3850 capillary electrophoresis system (Instrument Specialties Company, Lincoln, NE). This system may be interfaced to a personal computer using the software "Inject" (Bioanalytical Systems, Lafayette, IN) to collect and process data.

Another preferred capillary electrophoresis system is constructed using polyamine-coated, fused silica capillaries (Polymicro Technologies, Phoenix, AZ) of 50 μm inner diameter, 360 μm optical density (OD), and 35-60 cm in length to prepare the columns. The separation length is varied from 15-40 cm. Detection is achieved with a variable wavelength UV absorbance detector (Model V4, Instrument Specialties Company, Lincoln, NE). Protein elution is monitored at 200 nm and the product is monitored at the appropriate wavelength; e.g., NADPH is monitored at 340 nm. The neutral marker mesityl oxide is detected at 254 nm. Strip chart recordings are obtained with a Linear 2000 recorder (Linear, Reno, Nevada).

Reagents, e.g., substrates, cofactors, enzymes, or markers may be obtained from any pharmaceutical or chemical company, e.g., Sigma Chemical Co. (St. Louis, MO), Aldrich (Milwaukee, WI), or Calbiochem (San Diego, CA). In the example described in detail herein, Glucose-6-phosphate dehydrogenase reagent and G-6-PDH substrate solutions were purchased from Sigma Chemical Co. Reagents were prepared and the assay carried out as described in Lohr et al., Glucose-6-Phosphate Dehydrogenase, in: H. U. Bergmeyer (ed.), Methods of Enzymatic Analysis, 2nd English edn.,

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Verlag Chemie, Weinheim and Academic Press, New York, 1974, pp. 636. Ethyleneglycol diglycidylether (EGDE), 3-glycidoxypropyltrimethosysilane (GOX), 1,4-Diazabicyclo[2.2.2]-octane (DABCO), mesityl oxide, solvents and buffers were obtained from Aldrich Chemical Co. (Milwaukee, WI). Buffers were prepared with deionized, double distilled water.

Protein samples may be injected into the capillary electrophoresis apparatus by syphoning. The inlet end of the capillary may be inserted into the protein sample and raised approximately 5 cm for 5 seconds. Neutral marker is introduced into the capillary in the same way. The running buffer may contain all of the reagents necessary to assay enzyme activity. Assay reagents are reconstituted according to the reagent supplier (Sigma Chemical Co., St. Louis, MI) (Jorgenson et al., 1982, Analytical Chemistry 53:1298). For example, for the G-6-PDH assay described herein, the buffer solution contained 0.7 mmol/L glucose-6-phosphate (G-6-P), 0.5 mmol/L of the coenzyme nicotinamide dinucleotide diphosphate (NADP) and 4 mmol/L maleimide, in addition to a stabilizer and lysing agent. Operating current was controlled within the range from 35 to 50 μ A by limiting the applied potential, and was not operated above 60 μ A. All assays were carried out at ambient temperature without temperature control. Capillaries were cleaned by flushing with 0.01 M sodium hydroxide, double-distilled water, and then the working buffer solution.

Deactivation was achieved using a covalently bonded epoxy polymer layer. This coating has been shown to give greater than 95% recovery of proteins in capillary electrophoresis. Electro-osmotic flow in these deactivated capillaries is substantially reduced and negatively charged

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species, such as NADPH, can require 20-30 min. to pass through a 30 cm capillary. Because G-6-PDH has a relatively high pI value, it is transported quickly. The electrophoretic mobility of G-6-PDH and NADPH were found to be 5 and 18 min. respectively.

A capillary zone electrophoresis system was used in which the buffer tanks and capillary had been filled with running buffer containing all of the reagents necessary for the assay except the enzyme. Sample enzyme was introduced into the capillary by injection and potential was applied to initiate electrophoretic mixing of the enzyme and substrate. Product formation was measured with a UV detector at 340 nm. Assays were carried out separately under a constant potential mode, in which the reactants remained under constant potential throughout the time-course of the assay, and a zero potential mode, in which the separation of reactants was stopped during part of the assay.

The electropherogram in Figure 9(a) of a G-6-PDH assay obtained under constant potential has the general shape predicted in Fig. 3 for an enzyme having a greater transport velocity than the product of the reaction it catalyzes. The total time required for the assay in this case is slightly less than 12 min. When a longer capillary was used with a more dilute solution of enzyme, Fig. 9(b) shows that plateau height is lower and the elution time of the enzyme is longer. Data collection was terminated in this case before the trailing injection artifact peak eluted. (The height of the injection artifact peak was variable and of no analytical value.) The size of the injection artifact peak was found to be related to three factors: the size, volume and degree of mixing during the injection; enzyme concentration in the sample; and the time elapsed between injection and the start of electrophoresis.

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An example of an electropherogram in which several minutes was allowed to elapse between injection of the sample and the start of electrophoresis is shown in Fig. 10(a). For comparison, the electropherogram shown in Fig. 10(b) was produced as a results of an assay in which sample was injected quickly and the potential dropped to zero for several minutes before the enzyme passed the detector. The large peak at approximately 8 min. is the result of a several minute interruption in the potential, 5 min. into the run.

Analysis time also could have been decreased below 12 min. without loss in sensitivity by shortening the capillary. The optimum length in terms of minimizing analysis time would be the length required for the product elution curve to plateau. Increasing the potential to shorten analysis time was found to be counter-productive, as increasing the potential diminishes product accumulation and sensitivity.

The small peak eluting at 3.5 min. and 6.0 min. in Figures 10(a) and 10(b), respectively, adsorbed at 200 and 340 nm, and may be a protein in the sample that either adsorbs at 340 nm or binds NADPH but does not play a role in catalysis.

Switching the potential to zero before the enzyme elutes from the capillary allows one to increase the incubation time and thus the sensitivity. Zero potential assays were carried out in a 41 cm segment of capillary for a zero potential pause of up to approximately 6 min. The potential was interrupted after 3 min. and held at zero potential for 5 min., after which the potential was returned to 8700 V for

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product elution. An electropherogram of a sample estimated to contain 4.6×10^{-17} moles of G-6-PDH is shown in Fig. 11. This estimate is based on the assumption of a 2 nl injection volume. Note the peak at 20 min. which resulted from the 5 min. zero potential incubation. The dose-response curve for G-6-PDH using this assay procedure is seen in Figure 12. Data for this Figure were obtained from Table 2. The G-6-PDH assay shown in Table 2 was performed using a 17.8/41 cm GOX-EDGE coated capillary (50 μ m internal diameter). The 10 power supply was set at 8700 volts, 50 μ A. Sample was injected by raising the cathode end of the capillary tube for 10 seconds. Detection was carried out at 340 nm at a sensitivity of 0.02 au. Quantitation is based on determinations of the peak area above the constant potential 15 product plateau in the electropherogram. A solution containing one unit/ml of G-6-PDH is approximately 3×10^{-8} M. No attempt was made to determine the linear dynamic range of the assay, but similar macroscopic assays suggest that it may be two orders of magnitude (Sigma diagnostics 20 procedure No. 345-UV, 1990).

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TABLE 2

G-6-PDH ACTIVITIES OBTAINED BY CAPILLARY ELECTROPHORESIS ASSAY

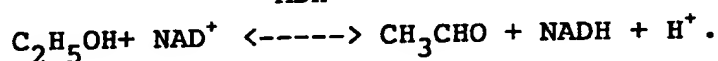
5	Assay Number	G-6-PDH activity	G-6-PDH concentration (Molar)	Quantity of G-6-PDH (moles)	Peak Area (Arbitrary)
10	1	0.75	2.3×10^{-8}	4.6×10^{-17}	85,241
	2	1.50	4.6×10^{-8}	9.2×10^{-17}	164,554
15	3	3.75	1.15×10^{-7}	2.3×10^{-16}	386,763
20	4	7.50	2.3×10^{-7}	4.6×10^{-16}	760,207

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2. Analysis of the Substrate Ethanol

The substrate ethanol was assayed using the capillary electrophoretic method of the invention. Substrate was determined after its conversion by the enzyme alcohol dehydrogenase (ADH, EC 1.1.1.1) to product. ADH catalyzes the oxidation of ethanol to acetaldehyde:

ADH



10

The concurrent reduction of NAD^+ to NADH can be directly monitored by the increase in absorbance at 340 nm. The equilibrium, which lies far to the left at neutral pH, can be forced to the right by buffering at pH 9.0 and by trapping the acetaldehyde with an agent, such as hydrazine. In the following experiment, the quantity of ethanol in the sample was measured.

The assays were carried out using non surface-modified polyimine-coated, fused silica capillaries (Polymicro Technologies, Phoenix, AZ) of 50 μm inner diameter and 360 μm outer diameter of various separation and total lengths. Detection was achieved using an ISCO (Lincoln, NE) CV capillary electrophoresis absorbance detector at 360 nm. A Spellman Model FHR 30P 60/EI (Spellman High Voltage Electronics Corp., Plainview, NY) power supply was used to apply the electric field across the capillary. Data was collected either on a Linear (Reno, NV) strip-chart recorder or on an i486 personal computer interfaced with a PC-LPM-16 I/O board and NI-DAQ DOS software (National Instrument Corp., Austin, TX). The spectrophotometric determinations were performed using a Spectronic 20D (Milton Roy Co., Niagara Falls, NY) spectrophotometer at 360 nm.

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Yeast alcohol dehydrogenase, NAD, glycine buffer solution, and p-nitrophenol were purchased from Sigma Chemical Co. (St. Louis, MO). The electrophoretic running buffer was prepared by diluting the 0.500M glycine buffer solution (containing hydrazine trapping agent) by a factor of 10 with degassed double-distilled-deionized water. ADH and NAD were dissolved in the diluted buffer solution to make the reagent/buffer solution. Absolute ethanol was purchased from Midwest Solvents Co. (Perkin, IL). Ethanol standards were prepared by diluting ethanol with appropriate amounts of double-distilled-deionized water and adding p-nitrophenol as an internal standard. Mesityl oxide (neutral marker used in determination of electrophoretic mobilities) was purchased from Aldrich Chemical Co. (Milwaukee, WI).

15

The capillaries were treated with 1N NaOH for 10 minutes and then rinsed with buffer solution for 10 minutes prior to use. The capillary and buffer reservoirs were filled with buffer/reagent solution. A plug of ethanol was injected into the cathodic end of the capillary by siphoning for a fixed time. The assay was effected by applying a potential and monitoring the absorbance electropherogram at 360 nm. All assays were performed at ambient temperature without temperature control. The spectrophotometric determination of ethanol was carried out as described in Sigma Diagnostic Procedure 332-UV, 1990, hereby incorporated by reference.

For assay of the substrate ethanol, a plug of the ethanol was injected into an uncoated capillary filled with pH 9.0 glycine buffer solution containing the enzyme alcohol dehydrogenase, coenzyme (NAD), and trapping agent (hydrazine). Ethanol is electrically neutral ($\mu_{em} = 0$ cm²/Vs), and thus moves with the electro-osmotic flow ($\mu_{eo} = 5.4 \times 10^{-4}$ cm²/Vs) toward the negative electrode. Alcohol dehydrogenase ($\mu_{em} = -1.6 \times 10^{-4}$ cm²/Vs) and NAD ($\mu_{em} =$

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-1.2x10⁻⁴ cm²/Vs) are each negatively-charged at pH 9.0, and thus electromigrate against the electro-osmotic flow; however, since the electro-osmotic flow is of greater magnitude, each travels toward the negative electrode and the detection window. In an electric field of 1500 v/cm, a 5 0.5 mm wide plug of ethanol will be fully interpenetrated with an adjacent alcohol dehydrogenase and NAD zone in less than 0.3 seconds, as shown in equation (4). As the enzyme and coenzyme electrophorese into the substrate's zone, 10 ethanol is oxidized to acetaldehyde, and NAD is converted to its reduced form, NADH ($\mu_{em} = -2.3 \times 10^{-4}$ cm²/Vs). As NADH is the only species present which absorbs appreciably at 340 nm, the gain in absorption at 340 nm as the NADH migrates past the detection window is a direct measure of the extent 15 of the reaction. If the width of the ethanol plug is assumed to be relatively narrow, the concentration of NADH produced at any given time is a direct measure of the instantaneous rate of the reaction of the ethanol with the enzyme. Since NADH has a lower electrophoretic velocity 20 than ethanol, the resulting electropherogram takes on the appearance of a rising plateau. A 0.5 mm wide plug of ethanol will encounter approximately 100 times its original volume in NAD and 150 times its original volume in alcohol dehydrogenase for a separation length of 25 cm (equation 25 (6)).

In order to accurately quantitate capillary electrophoretic assays, variations in reproducibility of injection volumes must not affect the accuracy of the 30 results. Where the quantity of injected substrate is determined rather than the concentration of substrate, an internal standard was used to measure the injection volume. p-nitrophenol was selected as the internal standard for this study as it exhibits high absorbance at 340 nm, and it is 35 sufficiently negatively charged at pH 9.0 ($\mu_{em} =$

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-3.2x10⁻⁴ cm²/Vs) to prevent comigration with the NADH product peak. The internal standard can be added to the running buffer, rather than the sample, and the resulting vacancy peak used as a measure of injection volume. This technique eliminates the need to reproducibly add the internal standard to samples, which can be quite difficult for microsamples.

Figure 13 shows a typical calibration curve for the capillary electrophoresis assay for ethanol. The experimental conditions for this particular calibration curve were as follows. The electrophoretic system included a separation length of 25 cm. The electric field was 1500 V/cm, and injections made by raising cathodic end of capillary 10 cm for 5 seconds. The ADH concentration was 75 units/mL, and the NAD concentration was 0.9 mM. The linear range extended to 4 g/L of ethanol; at concentrations above 4g/L significant truncation of the NADH peak occurred due to incomplete reaction of the injected ethanol. When forced through the original, linear regression yields a correlation coefficient of 0.9994. Five replicates of each standard determination were made. The relative standard deviations ranged from 3.0 to 5.4%. The lower limit of detection for this example was 0.01 g/L.

The linear and dynamic ranges of this technique are determined by the quantity of ethanol which can be oxidized prior to passing by the detector window. Thus, they are highly dependent upon the assay conditions chosen. The linear range of this technique has been extended to higher substrate concentrations by increasing the concentrations of enzyme and coenzyme in the running buffer thereby elevating the quantity of ethanol which can be oxidized prior to passing by the detection window. The injection of smaller volumes of substrate increases the linear range at the high

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concentration end, but results in a simultaneous increase in the lower limit of detection. Increasing the separation length of the capillary (at constant electric field) or decreasing the electric field (for a given capillary length) also expands the linear range by effectively increasing the reaction time, but result in a longer analysis time.

The reproducibility of the method as calculated in the determination of calibration curves (5 replicate trials per standard) typically falls in the range of 1 to 8% relative standard deviations. Greatest variability is found for low concentrations of analyte and enzyme as the resulting broad, flat peak profile makes it difficult to define the peak and baseline. Two determinations (1.0 g/L and 2.0 g/L) of higher replicates (n=10) produced relative standard deviations of 3.2 and 1.8%, respectively. The reproducibility of quantitative capillary electrophoresis performed according to the invention is limited by the inherent variability in electro-osmotic flow. The peak area is inversely proportional to the electro-osmotic velocity of the detected species as it passes the detector window. Electro-osmotic velocity is the sum of electrophoretic mobility and electro-osmotic flow. Thus, any variations in electro-osmotic flow will produce concurrent alterations in peak area. An internal standard may be used to resolve fluctuations which occur from one assay to the next. However, changes in electro-osmotic flow which occur during the detection process are not easy to resolve. These variations may be especially pronounced when the sample matrix contains components which are capable of adsorbing to the capillary wall thereby altering the zeta potential and resulting electro-osmotic flow, such as charged proteins. Samples such as blood specimens, which contain interfering components may require the use of surface-modified

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capillaries to prevent adsorption. Alternatively, sample preparation steps may be used to remove the interfering matrix components.

- 5 The capillary electrophoresis method of the invention was compared to a conventional spectrophotometric method of ethanol analysis. Ten samples containing between 0 and 4 g/L of ethanol were analyzed by both the capillary electrophoresis assay and the Sigma Diagnostics Alcohol kit.
- 10 The Sigma spectrophotometric method employs the same enzymatic reaction as the capillary electrophoresis assay. After ten minutes of incubation of the sample in pH 9.0 buffer containing ADH, NAD, and hydrazine trapping agent, the increase in absorbance at 340 nm is directly
- 15 proportional to alcohol concentration in the sample. Figure 14 compares the results of the two assays. Linear regression analysis of the data yields $y(\text{Sigma}) = 1.04 x(\text{capillary electrophoresis}) - 0.038 \text{ g/L}$, with a correlation coefficient of 0.995. The paired results of the eight
- 20 samples yielded a paired-t calculation of $t_{\text{calc}} = 0.284$ compared to a table value of $t_{0.950}$ of 2.365. These results indicate that the two methods yield similar values for the determination of ethanol. The reproducibility of the capillary electrophoresis method is also comparable to that
- 25 of the Sigma assay (4.1% and 1.08% relative standard deviations in two ethanol spiked serum pools).

- The specificity of the assay is consistent with that observed with all ADH assays. While ADH does not react
- 30 appreciable with methanol or acetone, it does react with certain alcohols other than ethanol. The rate of oxidation of these alcohols decreases in the following order:
ethanol = allyl alcohol > n-propanol > n-butanol > n-amyl alcohol > isopropanol. Although this lack of specificity is

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a disadvantage for all ADH-based assays, isopropanol is the only interfering alcohol which is encountered clinically. For the conditions previously described (pH 9, 60 μm inner diameter uncoated capillary, 25 cm separation length, 5 100 V/cm), the flow rate of buffer/reagent solution is approximately 0.38 $\mu\text{L}/\text{min}$. A typical 16 minute assay requires only about 6 μL of buffer/reagent solution containing about 5 nanomoles of NAD and 0.5 units of ADH.

10 Other embodiments are within the following claims.

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CLAIMS

1 1. A method of analysis of an analyte in a sample,
2 said method comprising
3 (a) introducing a reactant and a sample
4 comprising an analyte into a capillary containing an
5 electrophoretic running buffer wherein one of said
6 analyte or said reactant is electrically charged, and
7 wherein chemical contact between said analyte and said
8 reactant results in the breaking or formation of a
9 covalent bond in at least one of said analyte or
10 reactant to produce or deplete a detectable product;
11 (b) imposing along the length of said
12 capillary an electric potential for a time sufficient
13 to bring into chemical contact by electrophoretic
14 mobility within said capillary said analyte and said
15 reactant so as to induce formation of or to deplete
16 said product; and
17 (c) detecting said product.

1 2. The method of claim 1 wherein said capillary
2 used in step (a) contains reactant loaded thereinto
3 prior to the introduction of said sample.

1 3. The method of claim 1 wherein said electric
2 potential is imposed on said capillary for a time
3 sufficient to separate said product from one of said
4 analyte and said reactant prior to step (c).

1 4. The method of claim 1 wherein said electric
2 potential is imposed on said capillary for a time
3 sufficient to separate by electrophoretic motion said
4 analyte from other sample components prior to said
5 chemical contact between said analyte and said
6 reactant.

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1 5. The method of claim 1 including the additional
2 step prior to step (c) of:
3 (d) subjecting said capillary to zero
4 potential by removing said electric potential after
5 chemical contact between said analyte and said reactant
6 for a time sufficient to allow said product to form or
7 to disappear.

1 6. The method of claim 5 further comprising after
2 step (d) and prior to step (c) the step of
3 re-imposing along the length of said capillary
4 said electric potential for a time sufficient to allow
5 separation of said product from said analyte and
6 reactant.

1 7. A method of simultaneous analysis of plural
2 analytes in a sample, said method comprising
3 (a) providing a sample comprising first and
4 second analytes;
5 (b) introducing said sample and a reactant,
6 into a capillary containing electrophoretic running
7 buffer wherein one of said first or second analytes or
8 a said reactant possesses an electrical charge;
9 (c) applying an electric potential along the
10 length of said capillary for a time sufficient to bring
11 into chemical contact by electrophoretic motion within
12 said capillary a said reactant and a said first analyte
13 to produce or deplete a first detectable product, and a
14 said reactant and said second analyte to produce or
15 deplete a second detectable product, wherein said
16 electrochemical contact results in the breaking or
17 formation of a covalent bond of said first analyte or
18 said reactant, and of said second analyte or said
19 reactant; and
20 (d) detecting said first and second products.

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1 8. The method of claim 7 wherein said electric
2 potential is imposed on said capillary for a time
3 sufficient to separate by electrophoretic motion said
4 first and said second analytes prior to said chemical
5 contact with said first and second reactants,
6 respectively.

1 9. The method of claim 7 comprising the additional
2 steps, after step (c) but before step (d), of
3 subjecting said capillary to zero potential by removing
4 said electric potential for a time sufficient to allow
5 at least one of said first and second products to form
6 or disappear, and reapplying said electric potential
7 along the length of reapplying said capillary for a
8 time sufficient to allow separation of said first and
9 second products.

1 10. The method of claim 7 wherein said first and
2 second products are identical.

1 11. The method of claim 7 wherein said first and
2 second products are detectably distinguishable.

1 12. The method of claim 7 wherein said first and
2 second products have distinct electrophoretic
3 mobilities.

1 13. The method of claim 1 or 7 wherein said sample
2 and said reactant are spaced apart within said
3 capillary, and upon said application of electric
4 potential an analyte in said sample and a said reactant
5 migrate towards each other and into chemical contact.

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1 14. The method of claim 1 or 7 wherein said
2 analyte comprises an enzyme and said reactant comprises
3 an enzyme substrate.

1 15. The method of claim 1 or 7 wherein said
2 analyte comprises an enzyme substrate and said reactant
3 comprises an enzyme.

1 16. An analysis device comprising
2 (a) means defining a capillary,
3 (b) electrophoresis apparatus for imposing
4 electric potential across said capillary;
5 (c) means for electrically connecting the
6 capillary to the apparatus; and
7 (d) a reactant disposed within said capillary
8 which reacts with an analyte to form or deplete a
9 detectable product by breaking or forming a covalent
10 bond on at least one of said reactant and said analyte.

1 17. The device of claim 16, further comprising
2 (e) means for detecting said detectable
3 product.

1 18. An apparatus for electrochemical analysis
2 comprising
3 a capillary filled with electrophoretic buffer
4 and a reactant which reacts with an analyte to produce
5 a detectable product by breaking or forming a covalent
6 bond on one of said reactant or said analyte, and
7 means for imposing an electric field along the
8 length of said capillary.

1 19. The apparatus of claim 18, further comprising
2 means for detecting said product within said capillary.

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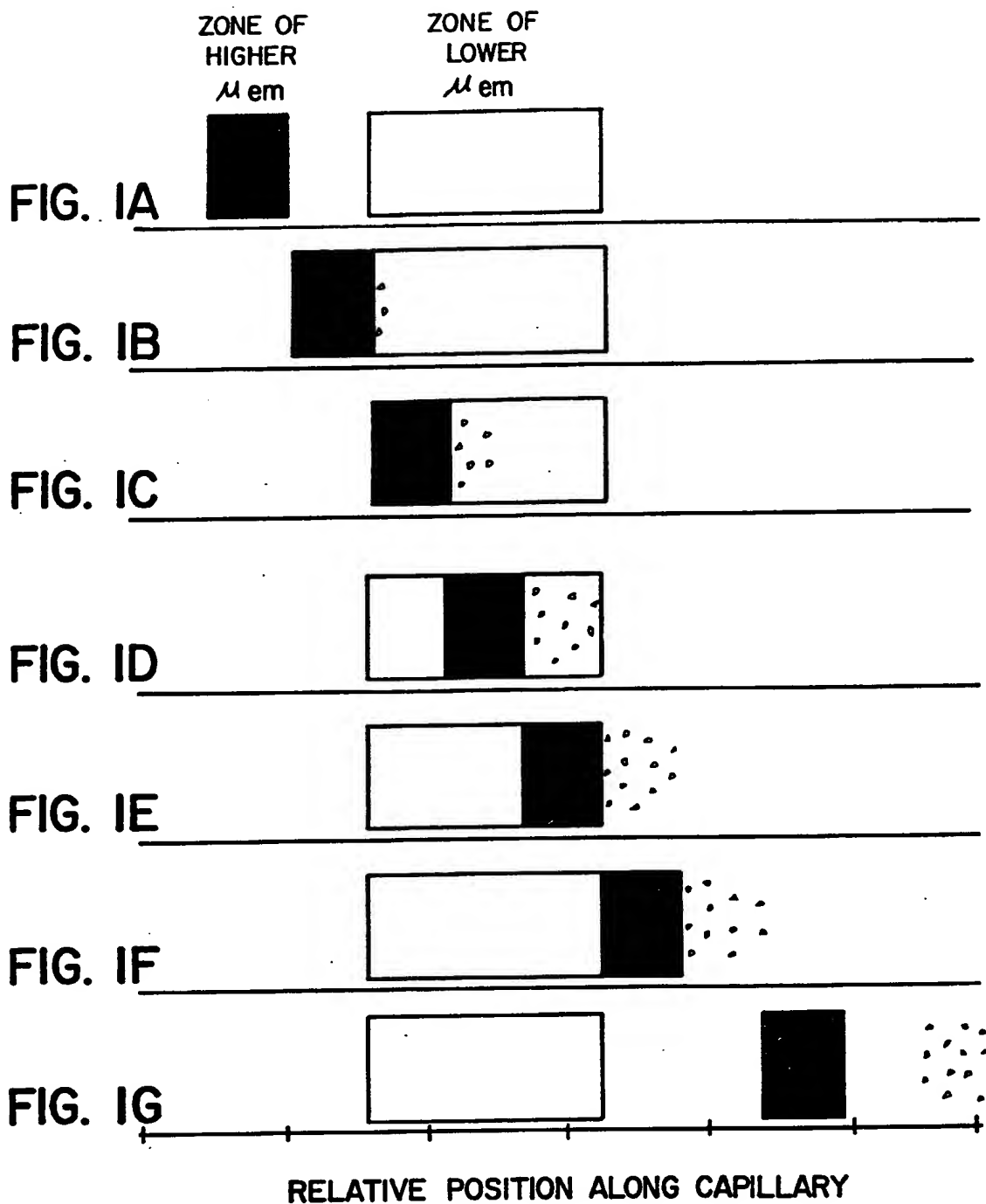
1 20. A capillary having a diameter of less than 500
2 microns and containing electrophoretic buffer, and an
3 analyte and a reactant spaced apart from each other,
4 wherein said analyte and said reactant are reactive
5 when in chemical contact to produce a product by the
6 breaking or formation of a covalent bond.

1 21. The method of claim 16, 18, or 20 wherein said
2 analyte comprises an enzyme and said reactant is a
3 substrate for said enzyme.

1 22. The method of claim 16, 18, or 20 wherein said
2 analyte comprises an enzyme substrate and said reactant
3 in an enzyme which converts said substrate.

1 23. The device of claim 16, the apparatus of claim
2 18, or the capillary of claim 20, wherein said
3 capillary comprises an interior charged surface.

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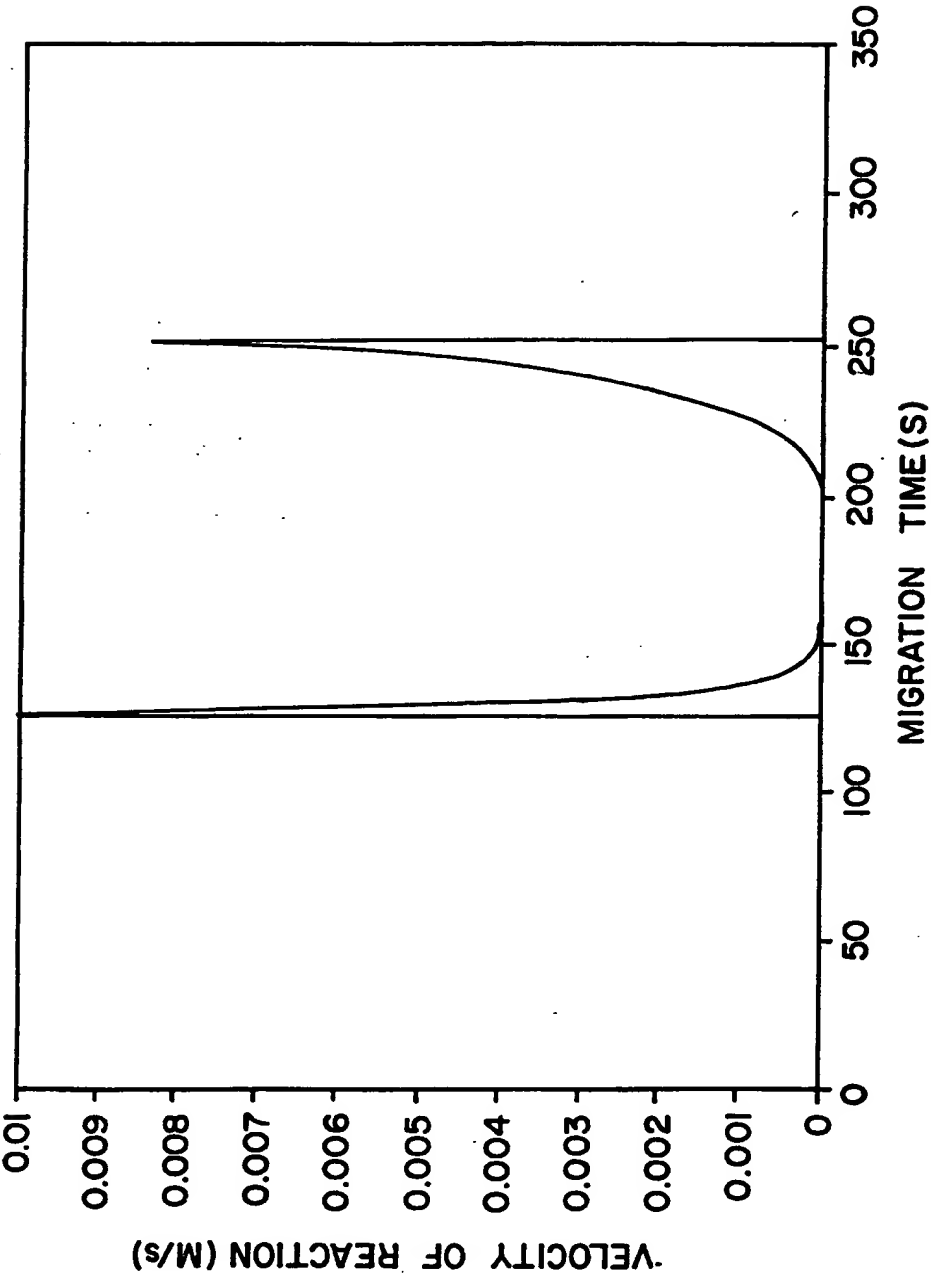
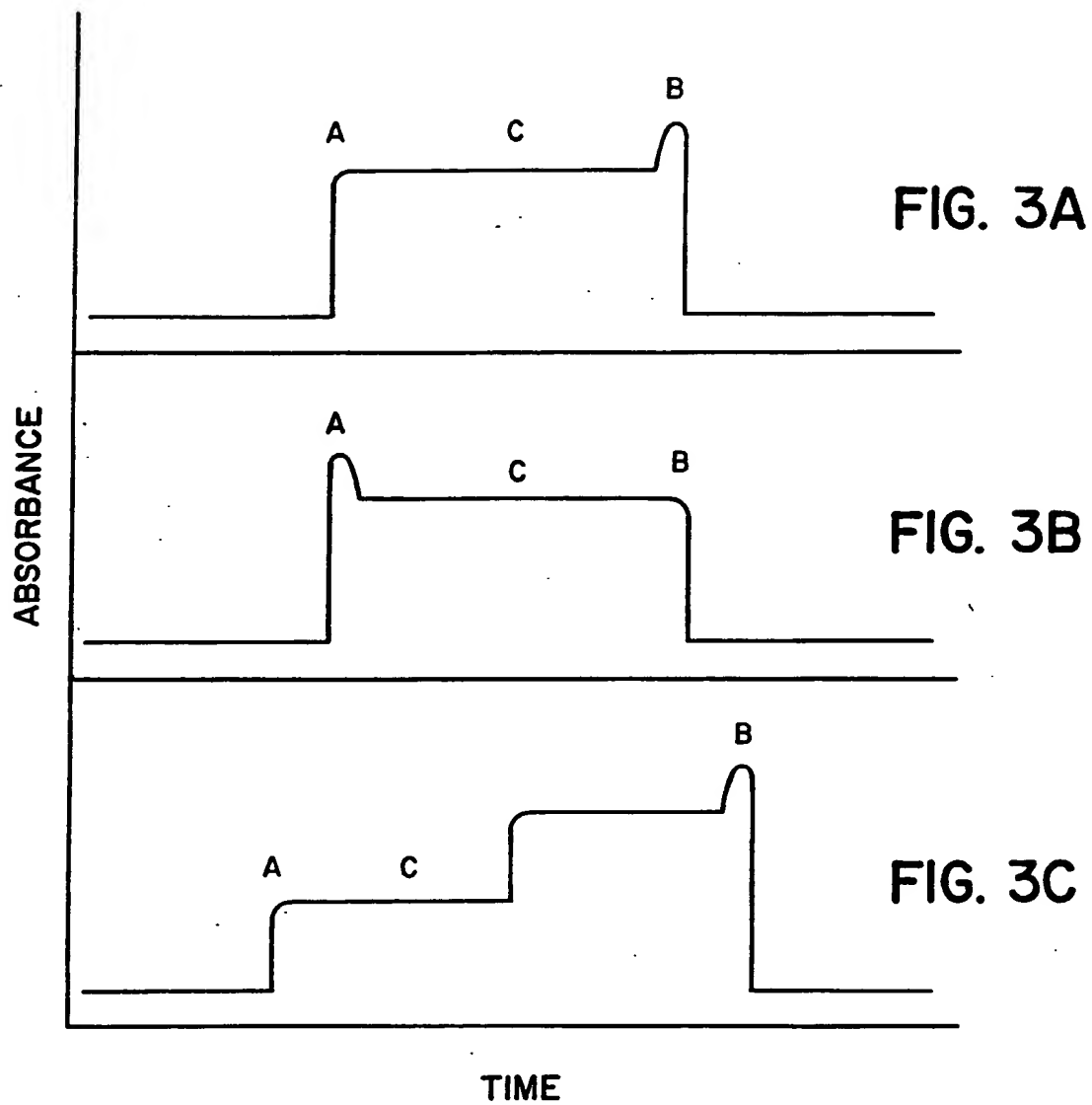


FIG. 2

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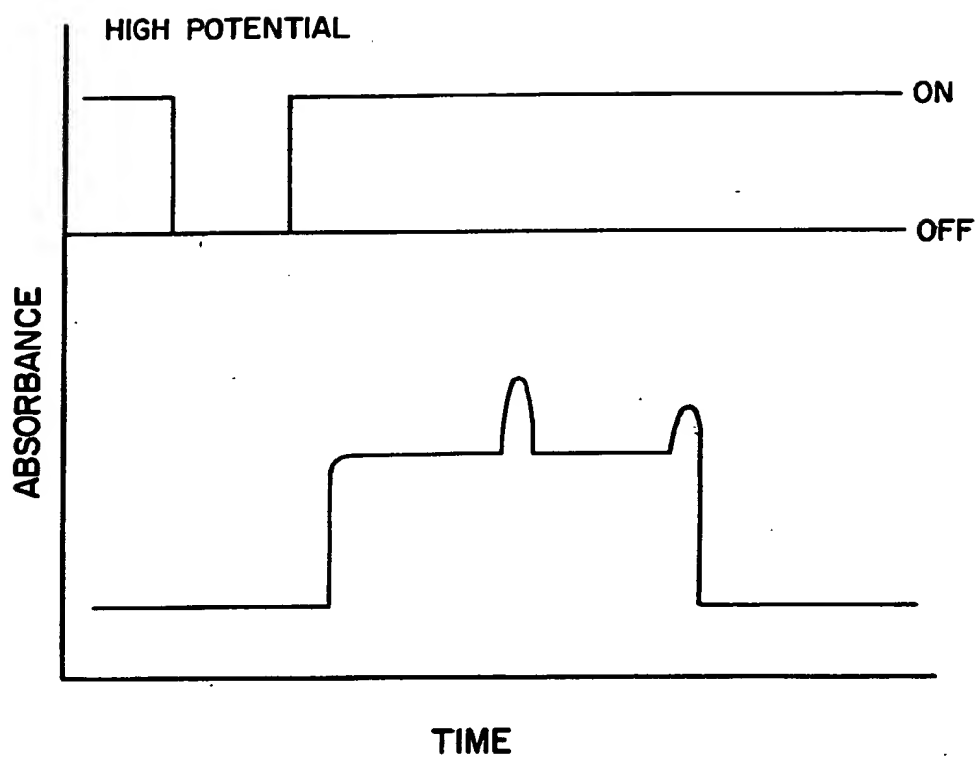


FIG. 4

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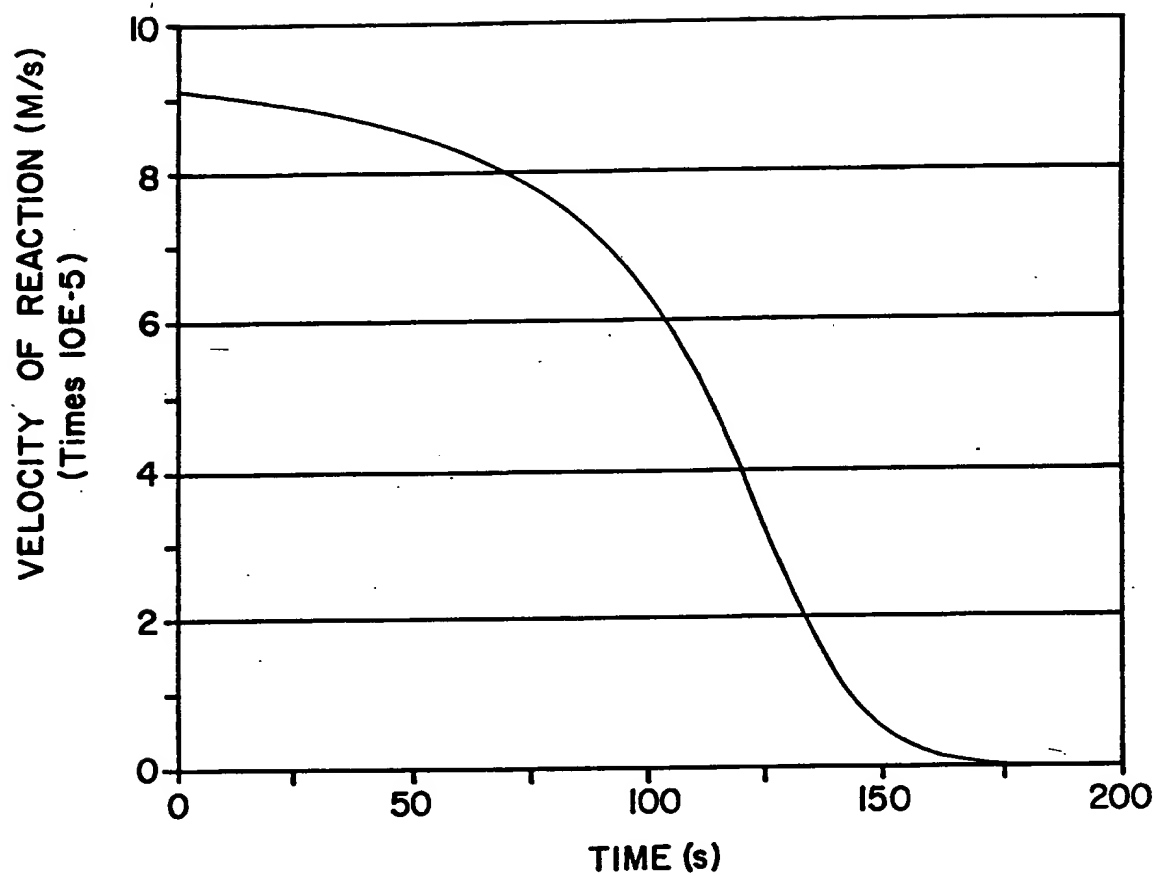


FIG. 5

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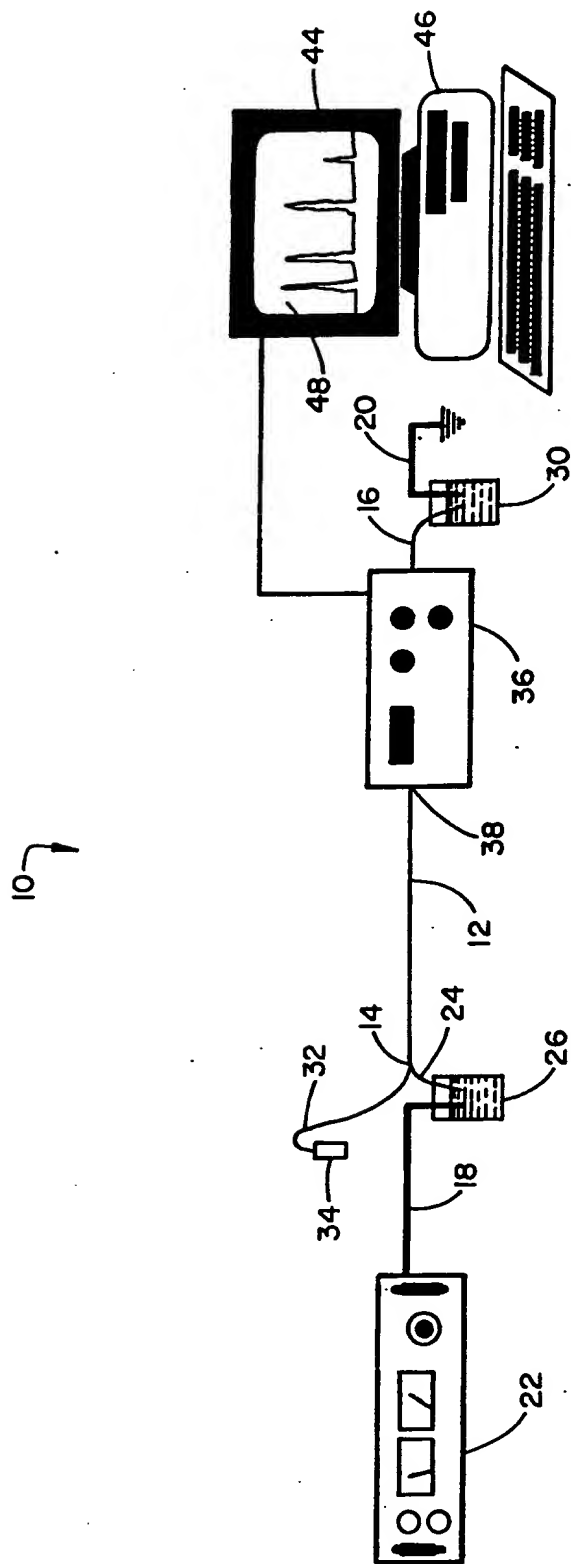


FIG. 6

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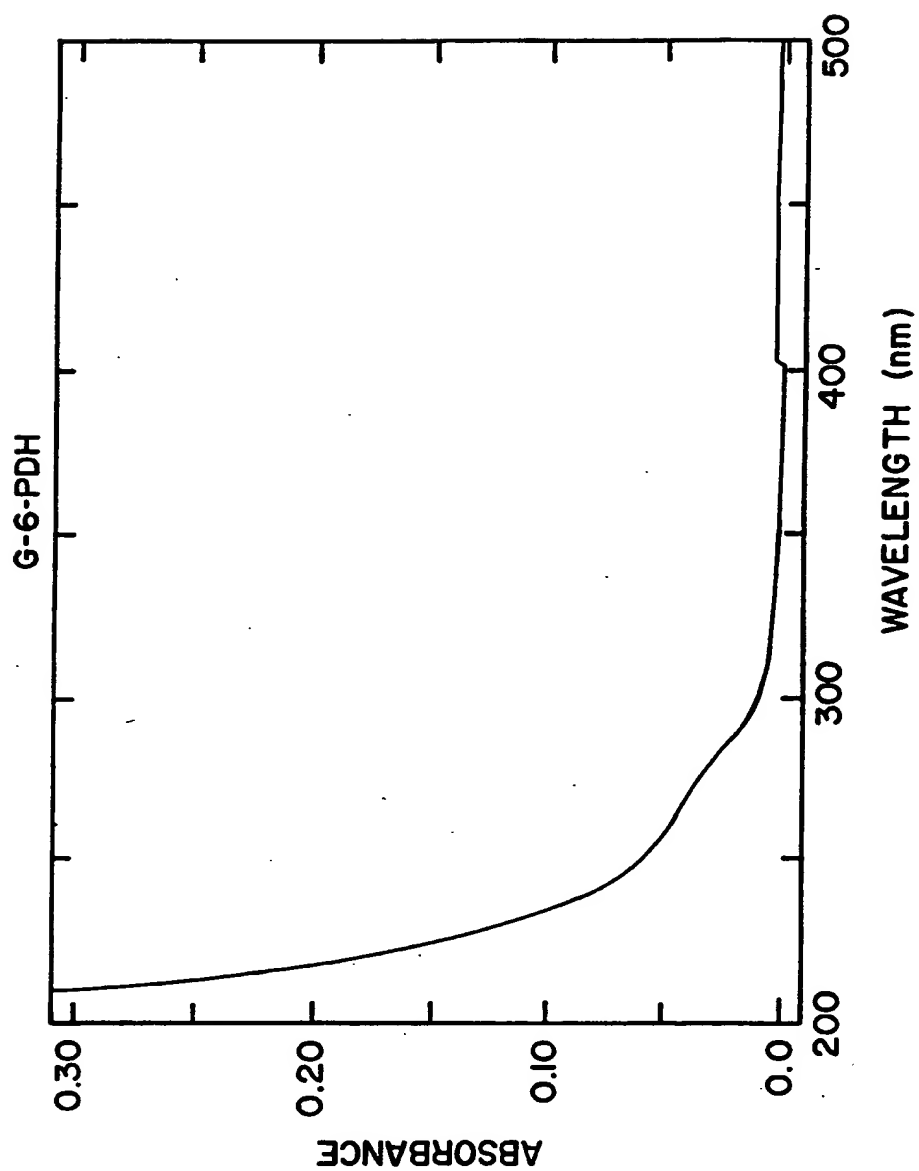


FIG. 7A

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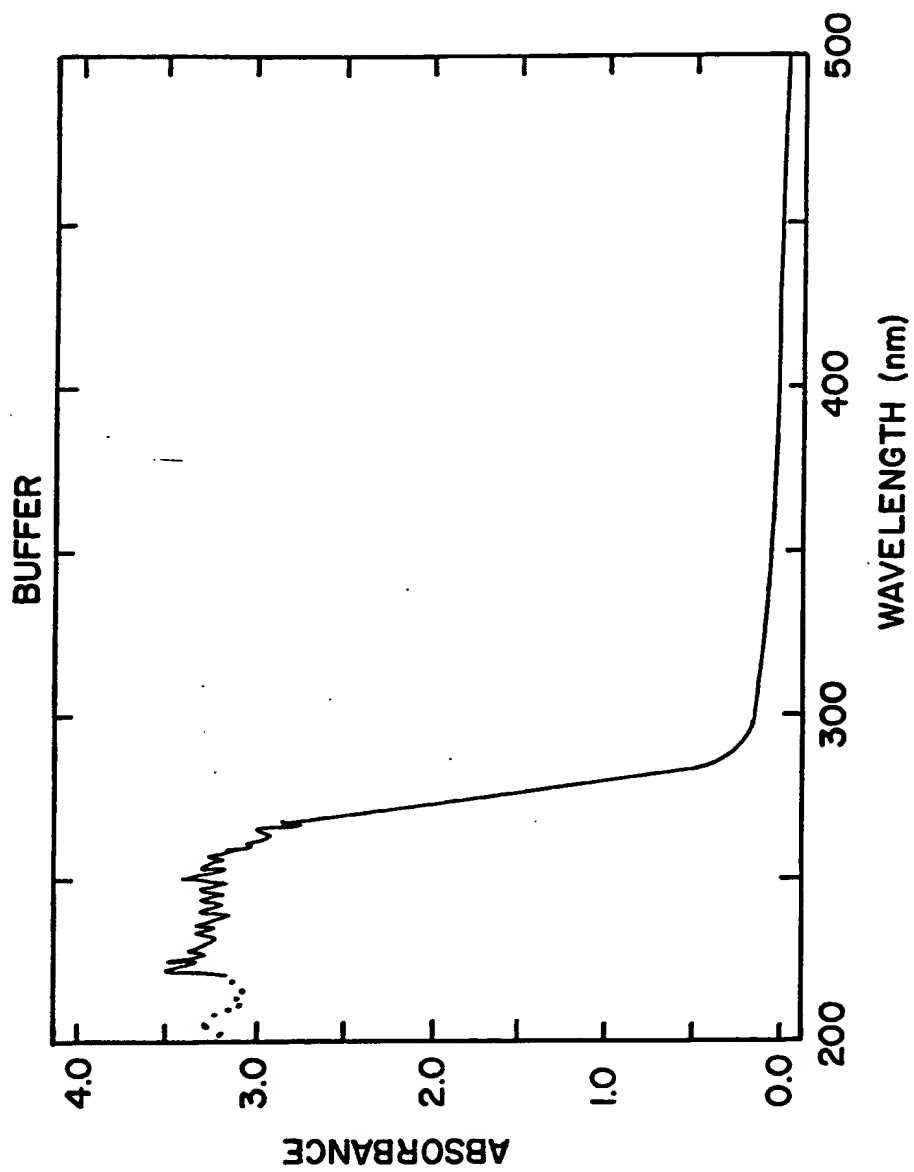


FIG. 7B

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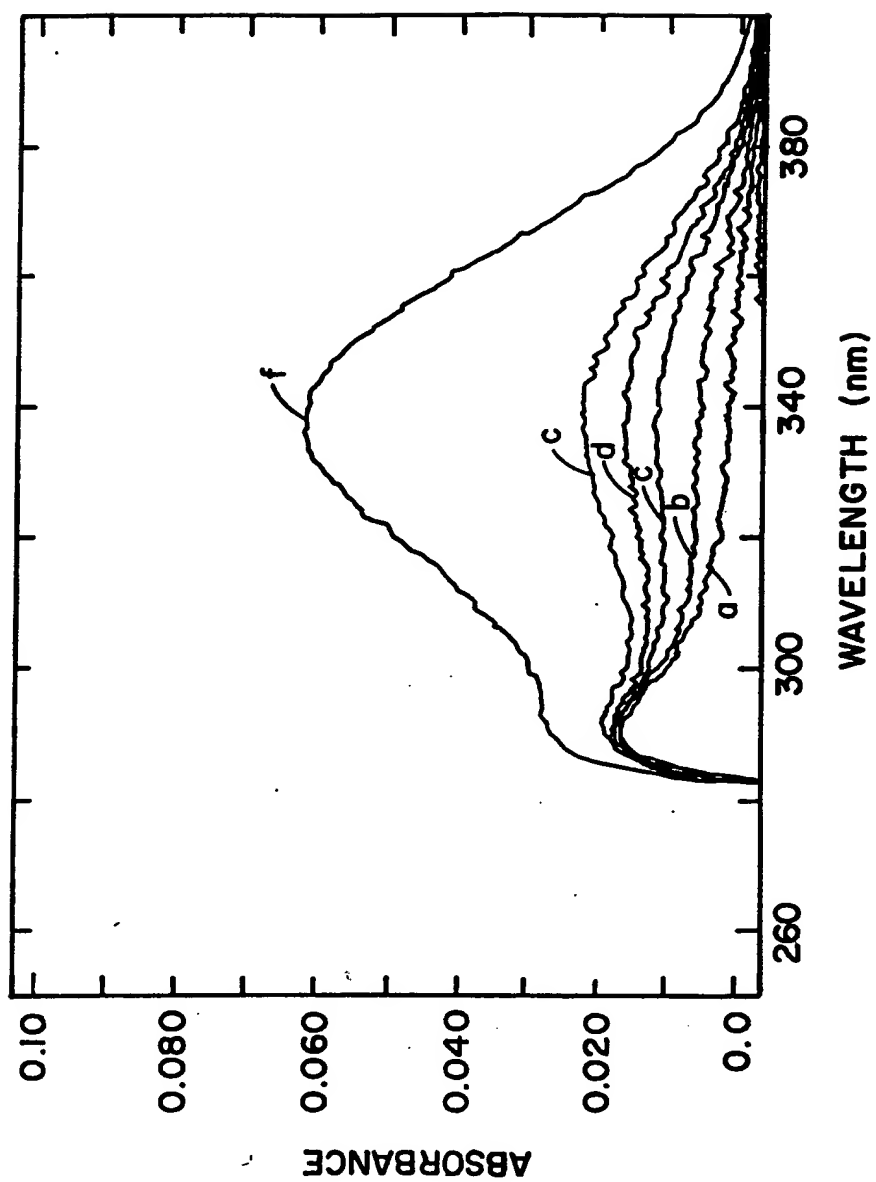


FIG. 8

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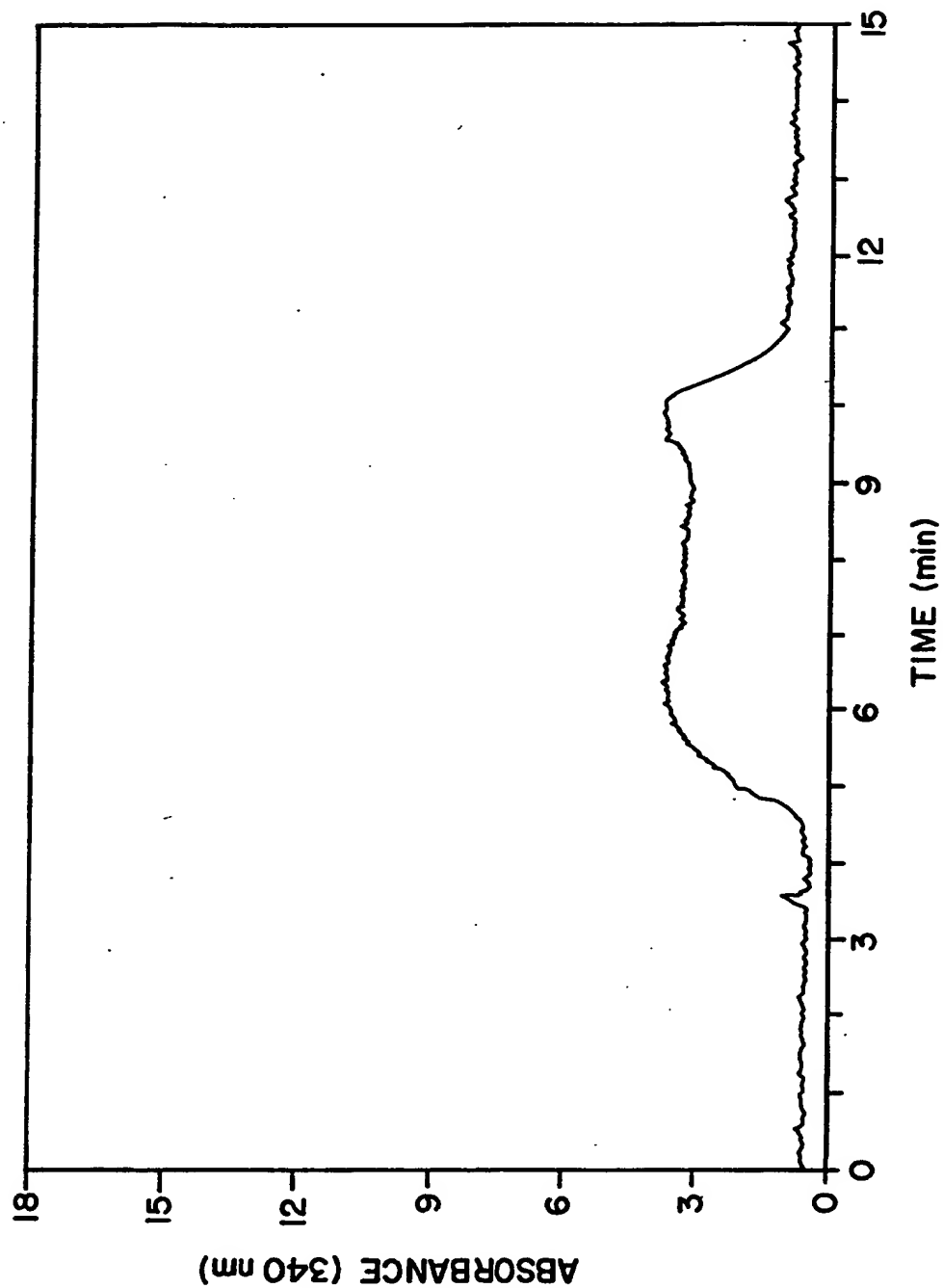


FIG. 9A

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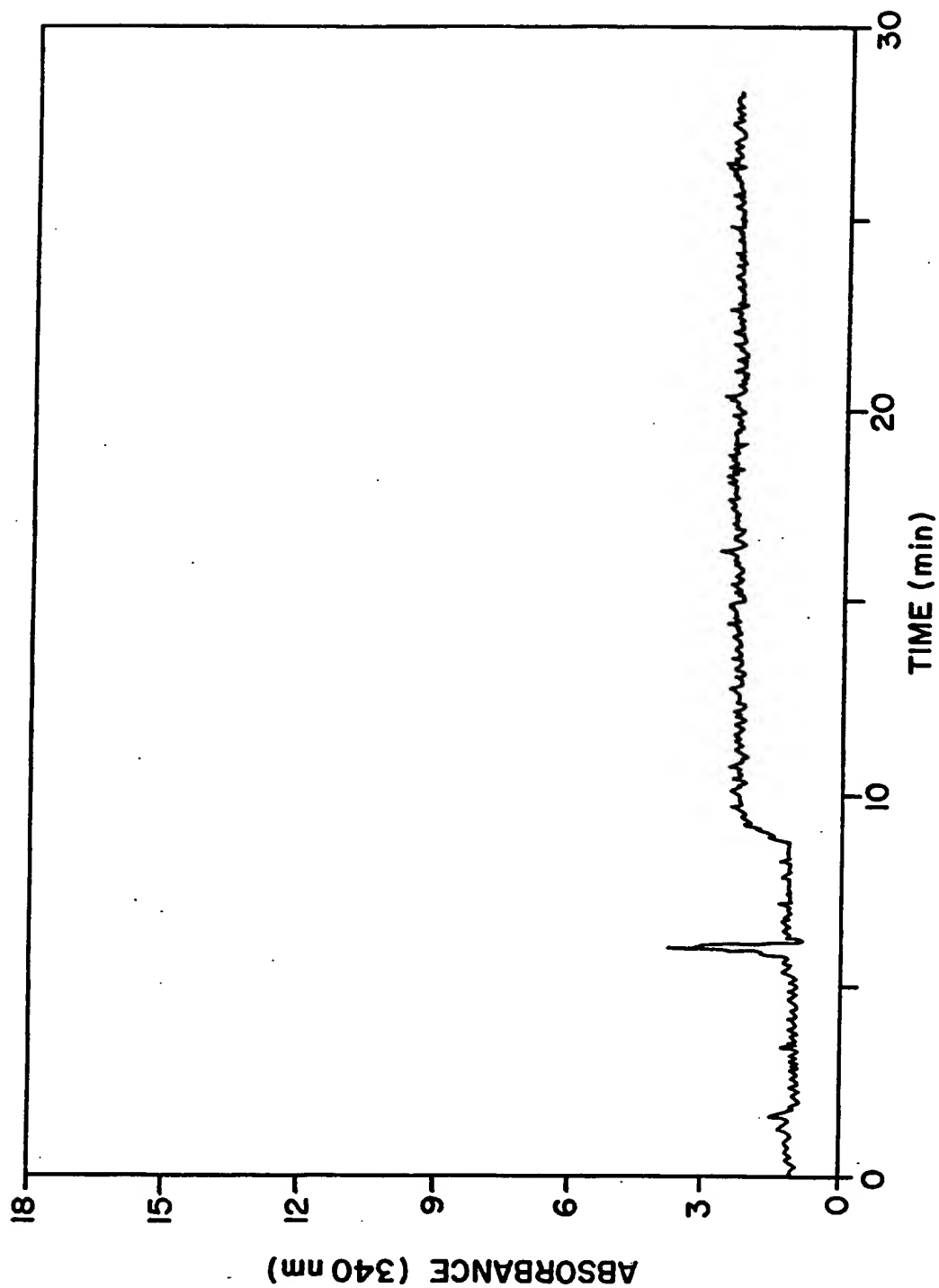
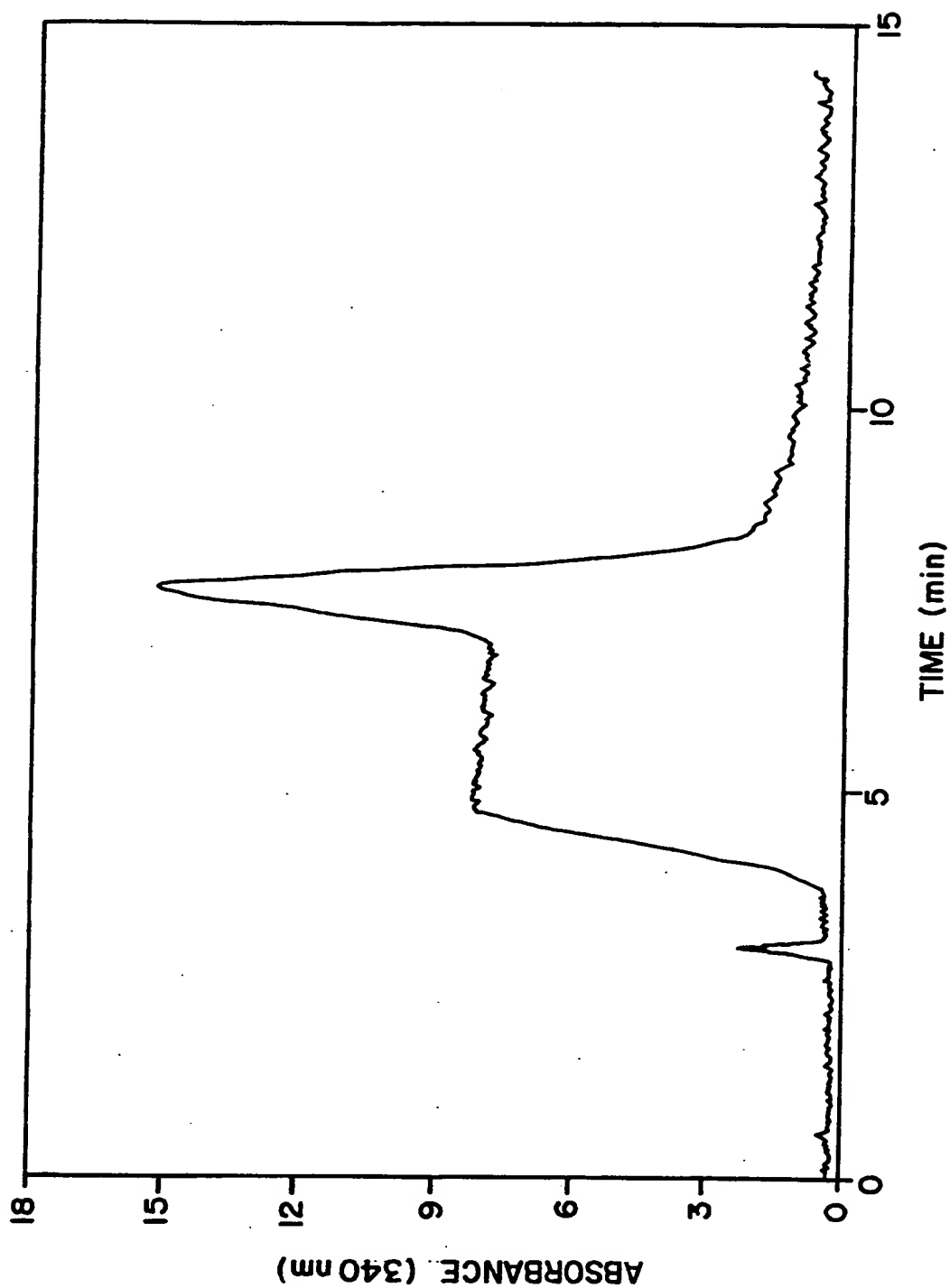


FIG. 9B

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TIME (min)

FIG. 10A

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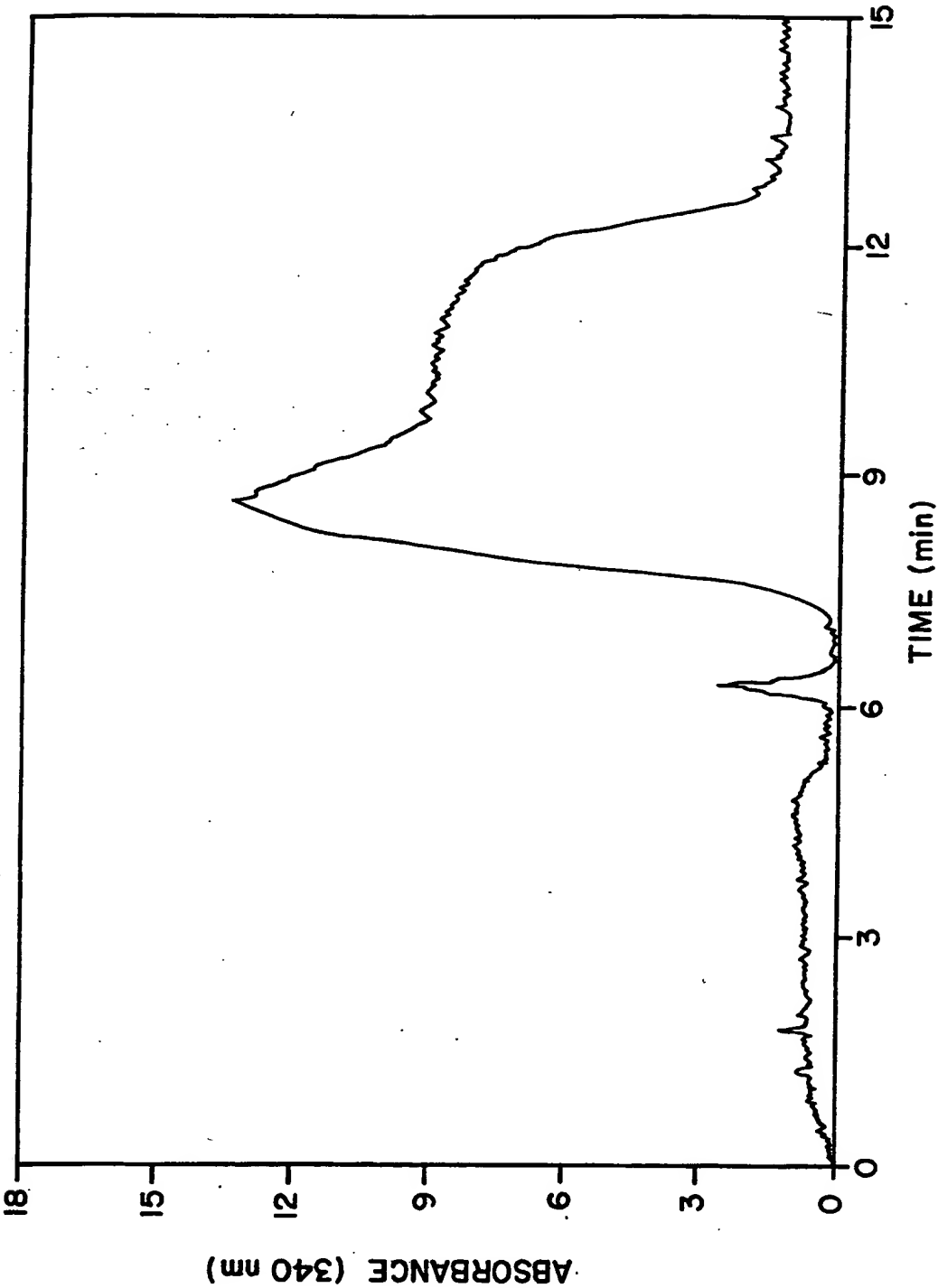


FIG. 10B

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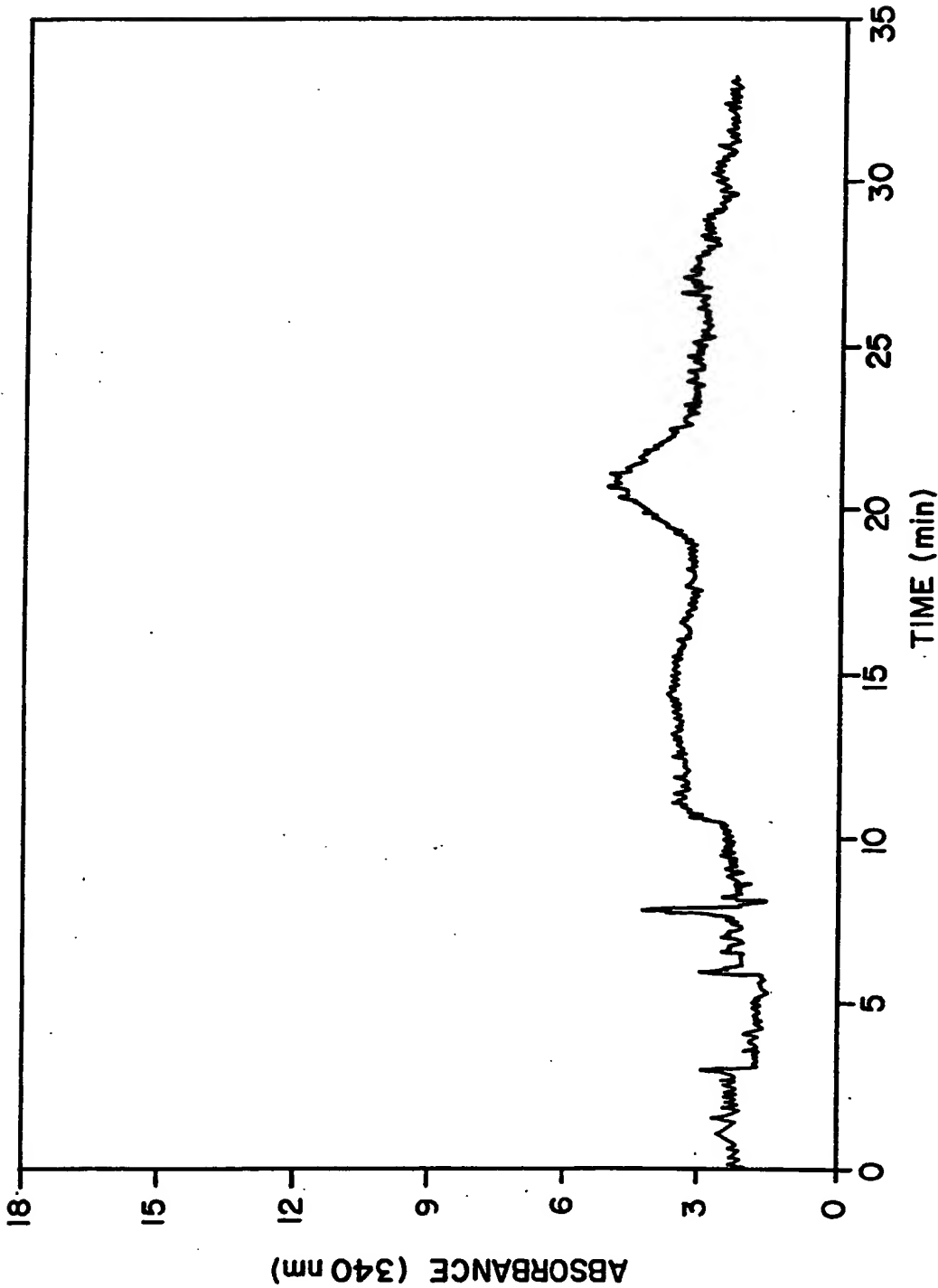


FIG. 11

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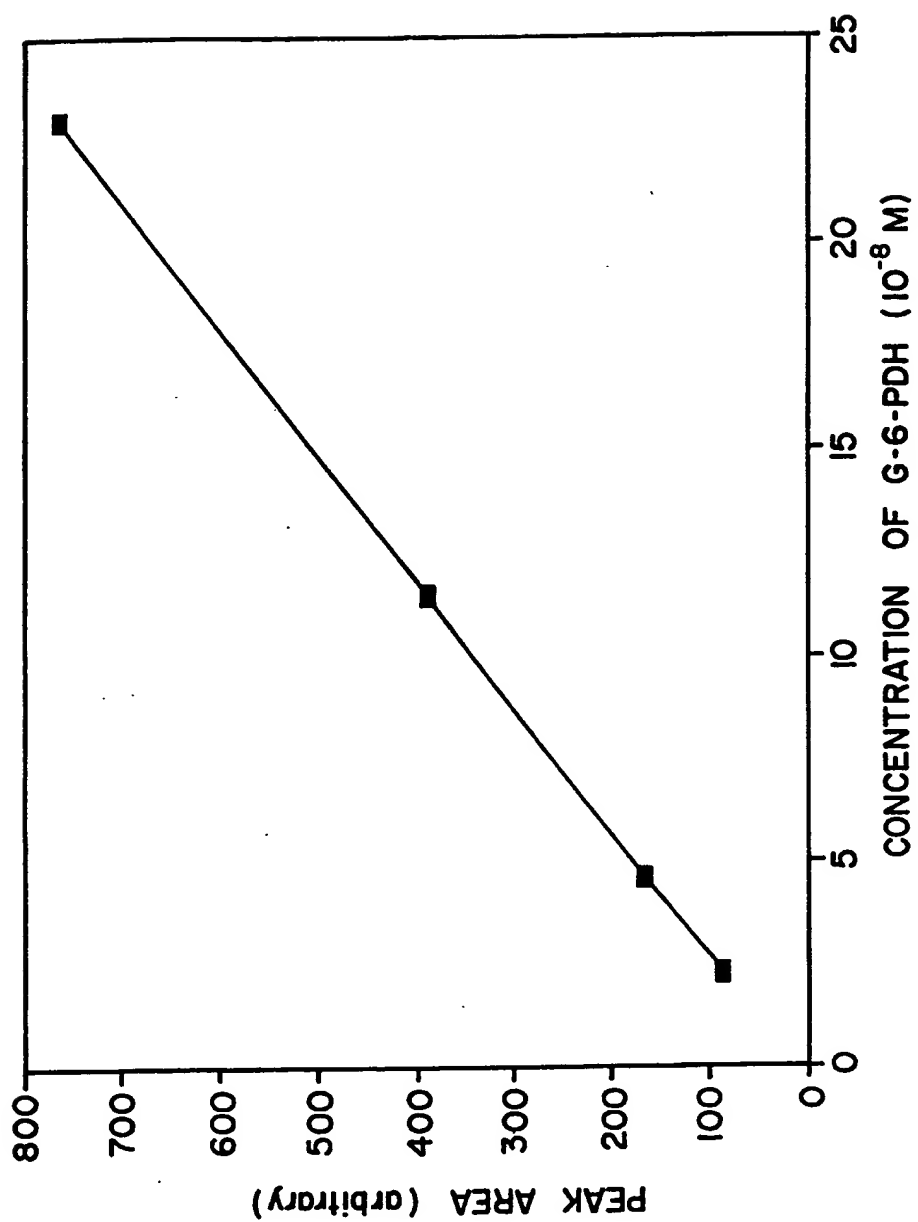


FIG. 12

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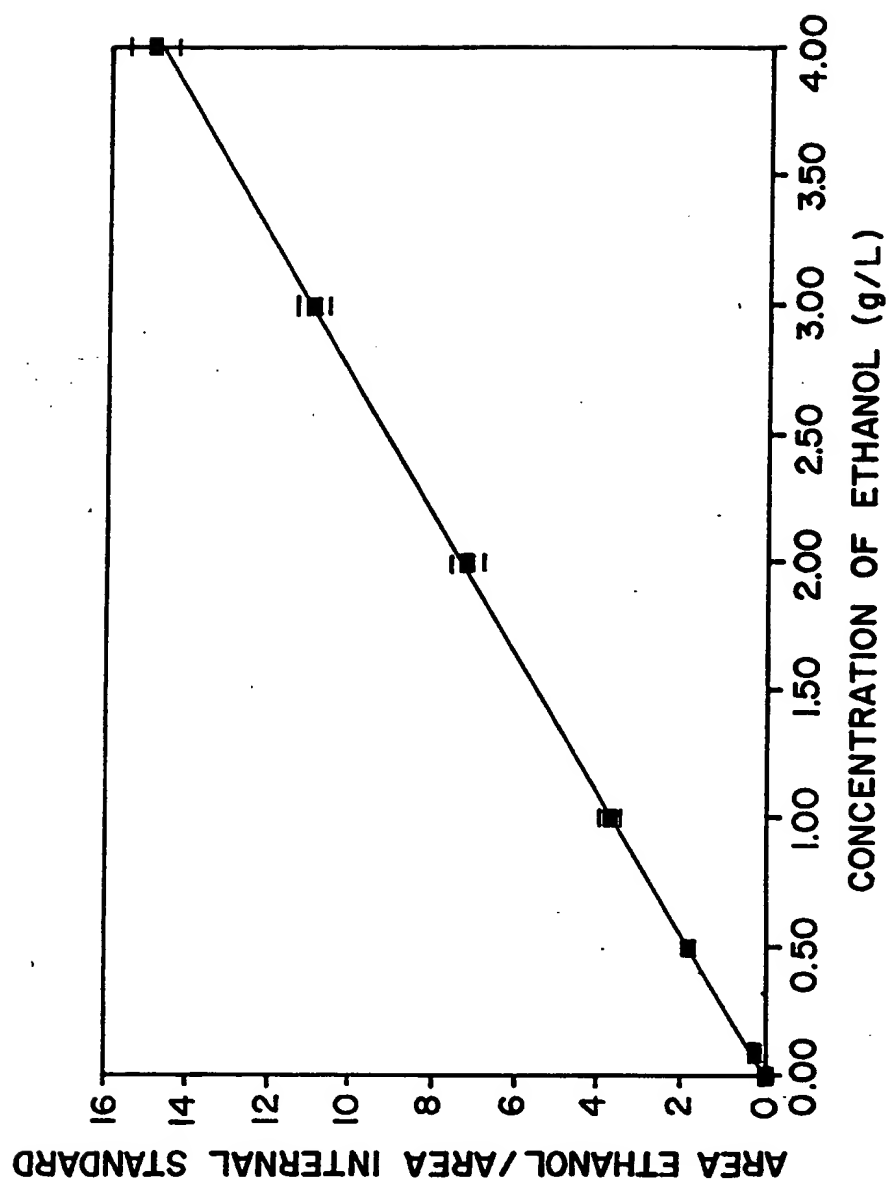


FIG. 13

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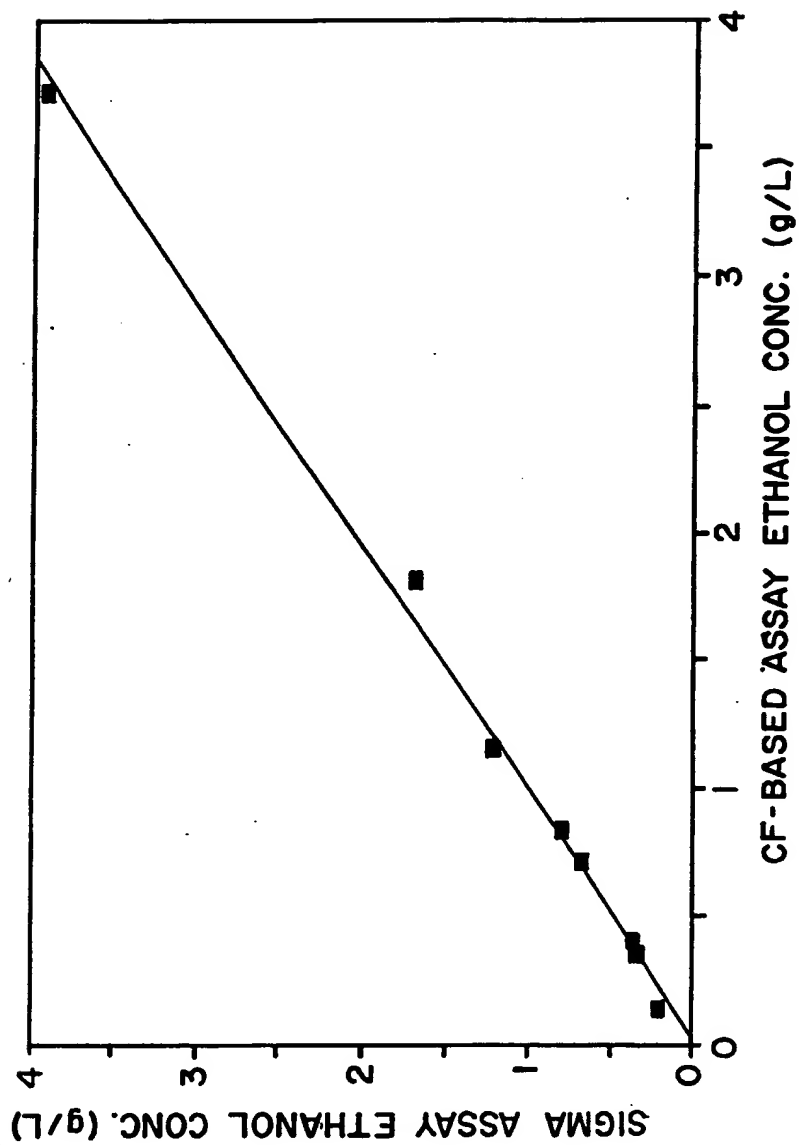


FIG. 14

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 93/08425

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 G01N27/447

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,91 06850 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 16 May 1991 see page 19, line 4 - page 30, line 26 ---	1
Y	US,A,5 145 567 (YOU ZUNG HSIEH) 8 September 1992 see column 4, line 32 - column 5, line 58; figure 1 ---	1
A	US,A,4 312 727 (J. R. SHAINOFF) 26 January 1982 see abstract; figure 1 ---	1
A	GB,A,2 040 313 (TECHNICON INSTRUMENTS CORP.) 28 August 1980 see abstract; figure 1 -----	1

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search

10 December 1993

Date of mailing of the international search report

21. 01. 94

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Authorized officer

Duchatellier, M

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 93/08425

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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